INNATE IMMUNE RESPONSES TO HEPATITIS C VIRUS

By

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To my dudes, Oguz, Osman and Kenan

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Hepatitis C Virus is a single stranded RNA virus that infects 170 million people world-wide and the risk of contracting HCV is higher in Hispanics individuals. In fact, Hispanics are Associated with an aggressive course of Chronic Hepatitis C Infection. It causes chronic liver disease and cancer in approximately 80 % of infected individuals. Currently, there is no vaccine available and current therapies to treat this virus are costly, lengthy (6-12 months), associated with significant side effects, and result in sustained viral response by only 50% of the patients. The fundamental question in HCV research is how the virus establishes a persistent and productive infection in the host where there is no generalized immune deficiency. Answers to this question may lead to an understanding of how the virus persists in an immune-competent host. A basic hypothesis is that HCV has developed strategies to evade the host's immune responses. The objective of this proposal is to elucidate the role of intracellular innate antiviral immunity in the co-evolution of HCV and host cells. If our central hypothesis is correct, we should be able to test novel approaches to shift the balance between HCV and hepatocytes in the setting of chronic infection toward an enhanced hepatocyte innate immune defense resulting in elimination of HCV from the human host.

CHAPTER 1
HCV AND INNATE IMMUNITY

1.1 Introduction

In 1989 a virus was discovered to be the cause of the blood-borne Non-A Non-B hepatitis infection that leads to liver disease (1-3). This member of the family *flaviviridae*, later termed Hepatitis C virus (HCV), infects approximately 210 people worldwide out of whom 80% will develop chronic infection (4, 5). This chronicity appears to be, at least partially, controlled by the host's immune response to the virus. It is the reason why therapies aimed at it, including the current one (pegylated IFN/Ribavirin), have often looked into preventing replication and jump-starting the immune response to induce clearance. However such therapies have proven ineffective in 50% of patients which highlights the need to not only understand the precise molecular mechanism behind these therapies but the reasons for non-response (6-8). For instance, this virus seems to interfere with the host's immune response without affecting its response against other pathogens. As a matter of fact, the role of type I interferon, the first line of defense, in natural HCV infection is not well defined. Currently, the fundamental question in HCV research is how the virus establishes a persistent and productive infection in the host where there is no generalized immune deficiency. Answers to this question may lead to a direct understanding of the interaction of HCV with its host and create the basis for therapies aimed at preventing and curing the disease.

1.2 Cellular Innate Immunity and Antigen Presentation in HCV

Several lines of evidence point at the importance the type of immune response plays in preventing or maintaining the chronic state of HCV. For instance, a bias towards type 2 responses has been shown to favor a chronic state in many diseases including HCV (9-12). *In vitro* studies have further confirmed this for HCV by showing that PBMC stimulated with viral

proteins are induced to secrete IL-10 and inhibit IL-12 (and hence IFNγ as well) (13). The reverse also seems to hold true since one of the mechanisms of action of the IFN/Ribavirin therapy is the suppression of IL-10 without affecting type 1 cytokines (14). In PBMC, the likely culprits for the shift in the T_H1/T_H2 balance are dendritic cells (DCs) as demonstrated by several observations: 1) DC have been found critical in directing the type of the antiviral response in chronic infections; 2) HCV patients with specific MHC II haplotypes are more likely to clear the infection; 3) A defective IL-12 production from patient's DCs was responsible for a defective antigen presenting response and IFNγ production; 4) Reduced DC percentages in HCV patients (although this can also be attributed to liver disease since there is an inverse correlation with serum ALT concentrations)(15-25). Therefore, there is a specific need to study the effect this virus has on these cells in order to further understand their role in the induced chronic state. Figure 1 compares the observed effects of HCV on different subsets of DC and related innate immune cells.

1.2.1 Impact of HCV on DCs

While still debated, more and more evidence seems to indicate that HCV has a direct impact on DCs which might be one of the primary places of viral escape before reaching its final destination in the liver. In general, data seems to drive the hypothesis that viral binding and/or entry (without the need for replication) can cause a myriad of "defects" in these cells (15, 19, 21, 26, 27). These are thought to contribute to the chronic state since clearance of the virus, either spontaneously or by therapy, restore cells to their normal state. Those defects include: low *in vitro* stimulatory capacity of patient DCs, lower expression of co-stimulatory molecules, decreased IL-12 secretion (with no change in IL-10), decreased type I IFNs and interferon stimulated genes (ISGs) expression and, as mentioned before, low absolute DC numbers in the

periphery (17, 20, 23, 24, 26). Apart from these characteristics, these cells show no differences in phenotype and functionality as compared to healthy controls (28, 29). Furthermore, these characteristics are virus-specific since the cells retain their ability to respond to other antigens such as influenza A, LPS and CD40L or to respond to the direct addition of activating cytokines like IL-2 or IL-12 (15, 16, 30-33). Furtheremore, pulsing cultured patient DCs with certain viral proteins correlates with the levels of IL-10 and TNFα as well as with the down-regulation of the expression of proteins in the NFκB pathway (11, 13, 16, 34, 35). Hence, the next question is to elucidate if it is a direct consequence of viral replication and at which stage does this happen (reviewed later).

In the case of viral replication, several experiments hinted at the possibility that HCV does not replicate on these cells and that the DCs from chronic patients that carried negative strand RNA on them cleared that genetic material when placed in culture (11, 16, 28, 29, 36, 37). Final confirmation came after the discovery that Claudin-1 was necessary for entry since DCs do not express it and it was then demonstrated that even pseudo particles expressing E1 and E2 are not able to enter (38). Therefore it is currently understood that HCV does not replicate in these cells indicating that the effects caused by the virus are a consequence of binding to DCs. DCs do express some of the binding receptors for the virus and viral particles have been shown to be internalized leading to viral protein presentation (39-41). There is also the possibility that some of these receptors might be the ones modulating the response by DCs, although based on the current data, this last possibility seems highly unlikely. A more likely scenario is that DCs are an intermediate that deliver the virus from the periphery to the liver since the HCV particles inside DCs are targeted to the non-lysosomal compartments where they are protected from degradation

(37, 42, 43). Therefore, there is a strong possibility that recognition through immune receptors can lead to immunomodulation favoring the pathogen.

1.2.1.1 Myeloid and Monocyte-derived DCs

Most of the research on DCs in the context of an HCV infection has been carried out on monocyte-derived DCs (MoDC) for their ease of use or from the isolation of total DC populations from peripheral blood. Most of these cells were derived directly from patients and it was not until recently when, with the discovery of the HCV strain JFH-1, most of the direct interactions of the virus with these cells began to be studied (44). These observations, made on total DCs, indicated that the phenotypes observed correlate strongly with those of myeloid or monocyte-derived DCs (MoDCs). For instance, chronic HCV patients have a reduced population of mDCs as compared to controls, defective allogeneic response by both mDC and MoDC, and are incapable of shifting towards pro-inflammatory responses (15, 18, 22, 25, 29, 45-48). Conversely HCV-mDC and HCV-MoDC differ in their ability to react to maturation stimuli. LPS improves the allostimulatory capacity of mDCs although not completely which had led many to the conclusion that there are no defects in these cells (27, 32, 33). MoDC from HCV patients do not behave this way and seem to remain partly immature after LPS maturation, likely due to an increase in IL-10 combined with a decrease in IFNα (16, 29, 37). Similar results are observed with other maturation stimuli like Poly (I:C) or TNFα (11). In general it seems that the immature cells are not able to up-regulate co-stimulatory molecules as compared to non-HCV DC and this may account for the lack of allostimulatory reactivity observed in these cells (48-50). More insightful information has been recently obtained *in vitro* with the aid of JFH-1 co-cultured with healthy DCs. The virus directly inhibited the up-regulation of co-stimulatory molecules without changing the expression of HLA-DR (37, 51). Interestingly, the sera of HCV

patients were able to reduce the latter when added directly to cultured MoDCs. In this setting pro-inflammatory cytokines were also absent while IL-10 increased, mimicking what was seen *in vivo* (37, 52). JFH-1 by itself was not able to inhibit LPS-induced maturation or cytokine production, in contrast to what was observed in MoDC derived from chronic patients (27, 32, 33, 38). We ourselves have studied the interaction of MoDC with JFH-1 at different stages of differentiation (monocytes, immature, LPS-matured) (51). We observed that monocytes do not differentiate into DCs upon stimulation and immature DCs remained low in co-stimulatory molecules just as what has been observed by others. Interestingly, the effect on mature DCs was a shift towards a type 2 response with higher IL-10 after infection and these effects were with the absence of viral replication. Most groups assumed that there were no effects on these cells due to the lack of phenotypical changes but instead they may be part of the inducers of a suitable environment for the virus. More reports on the direct interaction of the HCV with these cells should shed some light on the differences observed between the clinical observations and those made *in vitro*.

Transfection experiments on the other hand have also hinted at the possibility that some of the viral proteins themselves (core, NS3 and to a lesser extent the envelope proteins E1/E2) may be important in inducing the phenotype observed on mDCs, perhaps by recognition through TLR2 (35, 48, 52-58). Some of this research had been carried out when trying to harness the immunogenicity of these proteins in order to use loaded DCs in therapies against hepatitis C. In this particular setting they realized that while pro-inflammatory responses were induced, IFNα was severely impaired which had nothing to do with the maturation stage of these cells (11, 15, 52, 54-57, 59, 60). The normal phenotype was recovered once cells were treated with IFNα which may shed light on the reason behind the disparity observed in clinical samples of mixed

DCs (31, 52, 53, 61, 62). It may be that these proteins stimulate directly through TLR2 inducing the secretion of IL-10 which may induce some of the phenotypes in mDCs and MoDCs (63). Furthermore expression of TLR2 as well as MyD88 is enhanced in mDCs from HCV patients while preventing the induction of cytokines from these receptors (45). This over expression might also be induced by endogenous IFNα expression in these cells. Other TLRs do not seem to be involved in this pathogenesis but are capable of inducing others. For instance, TLR7/8 agonists impair monocyte-derived DC differentiation and maturation and, furthermore, the phenotype of TLR7/8 ligand-treated DCs is similar to DC defects found in HCV-infected patients (35, 64, 65). It seems clear that a better understanding of how innate receptors recognize and modulate the immune response will be of the utmost importance in HCV and may even carry over into other diseases as well.

1.2.1.2 Plasmacytoid Dendritic Cells

The strength of this DC subset lies in its strong ability to produce vast amounts of type I IFN, especially IFNα, in a short period of time. They express constitutively high levels of IRF-7 which is needed to produce the different types of IFNα (66). This cytokine can by itself enhance the expression of HLA class I and class II molecules as well as co-stimulatory molecules on immature DCs but not induce full maturation of mDCs which requires CD83 (14, 50). It acts by inducing IL-12 and TNFα without affecting IL-10. Interestingly, other reports have suggested that IFNα can have a negative effect on the induction of maturation of mDCs by some viruses, even though this has not been shown for HCV (47). This suggests that IFNα/Ribavirin treatment might in itself affect DCs and their function. Conversely, this same report also suggested that these observations might not be as relevant in this setting since when combined with Ribavirin they are known to enhance immune responses to HCV (64, 67). As a matter of fact responders in

this treatment induce ISGs, like 2^05^0-oligonucleotide synthetase, MX1, IRF7, and TLR7 genes as compared to poor responders. Due to the nature of IFNα ability to prevent HCV replication an understanding of the direct effects of the virus on these cells is therefore of the utmost importance.

Clinically, it seems that HCV does not affect the quantity of pDCs in peripheral blood or barely decreases them (25, 47, 49). At the same time, an increase in the numbers of pDCs in HCV^+ livers is observed probably due to the upregulation of RANTES (21, 64, 68, 69). This mobilization can be induced by either E1/E2 or core protein interactions demonstrated by a need for glycosilated HCV proteins which are needed for IFNα induction in these cells (64). Conversely, IFNα production in these cells could be downregulated via the induction of IL-10 and TNFα by myeloid cells (monocytes and immature mDCs) either directly or by the induction of pDC apoptosis (68, 70). Furthermore, the increase on this cytokines correlated with their concentration in HCV patient sera (71, 72). Overall, it seems plausible that a subsequent downregulation of IFNα takes place leading to many of the observations we described here since a lack of IFNα can induce DC survival as well as a maturation factor both of which are missing in HCV, including pDCs (49, 66). Furthermore, although the overall levels do not change, it does not preclude a high turnover rate in these cells due to the decrease in IFNα and hence the induction of apoptosis (68). Interestingly, contrary to mDCs and MoDCs, these cells did not increase HLA-DR after TLR-stimulation although co-stimulatory molecules did. Whether this occurs merely by a direct interaction of the virus with PRRs like TLR7 and 9 in pDC or by a bystander effect is not fully known but IFNα may be downregulated due to viral interaction with cell surface receptors. Interestingly, the data derived from patient cells did not correlate well with experiments carried out with JFH1 where the direct interaction of the virus with cultured pDC,

and not IL-10 and TNFα produced by monocytes, was the culprit in the lack of IFNα response by these cells (38). This contradicting result highlights the need for continued research in the area of HCV interactions with distinct DC subsets and their role in viral escape.

1.2.2 NK and Cytotoxic Cells

Recently a clearer understanding of the mechanisms of DC has demonstrated their direct interaction with cells of cytotoxic potential such as $CD8^{+}CD28^{-}$ T cells, NK and NKT cells and their role in the pathogenesis of disease (38, 73). Their interaction leads to NK cell activation as evidenced by the upregulation of MICA/B in response to IFNα which is impaired in chronic HCV (74).The quantities of these cells in the liver also tend to increase while needed T_H cells as well as the ratio of CD4:CD8 T cells decreases (75). They can also be implicated in the downregulation of type I IFNs by HCV or be directly affected by diminished IFN production mediated by decreased IL-15 production (74, 76). In this case, evidence indicates that a direct interaction of HCV-E2 with CD81 in the NK cell can impair its activation, cytokine production, cytotoxic granule release and proliferation (77-79). Furthermore, proliferation by these cells is impaired in HCV since NS5B modulates the cell-cycle progression in these cells leading to arrest (80). It is not clear whether a direct virus interaction or an interaction with affected cells (like DCs) diminish the activity of these cells but their cytotoxic potential makes them important targets for future studies, especially at the DC-NK interface and its role on HCV.

1.3 Hepatocytes as Key Inducers of Innate Immunity against HCV

Hepatocytes, the end host of HCV, are usually considered as a secondary in the immune fight against this virus as compared to immune residents of this tissue (81). A myriad of evidence is changing this image demonstrating that the initial innate response by liver cells can not only prevent viral infection but induce a state that would induce faster clearance in the event of

infection, even if the majority of the peripheral IFN comes from immune cells (73, 77, 81-83). Apart from their direct use against HCV, evidence for the importance of type I IFNs in the induction of an antiviral state in hepatocytes comes from *in vitro* as well as *in vivo* studies on chimpanzees (84). In both of these, there seems to be a direct correlation between IFN and viral levels (84-86). Furthermore, the fact that both levels fluctuate in the primates provides further evidence that the interaction of HCV with its host is more of an equilibrium that can change drastically under specific conditions. Both of these models lacked that critical evidence that would connect IFN with viral levels and illustrate the need to understand the underlying mechanisms of action in the body of the host.

Until recently, most of the data had indicated that hepatocytes were not inducing type I IFN in response to HCV. Further advances have demonstrated that this was probably due to the use of hepatoma cells as model systems since most of them have defects in the IFN pathway. For instance, Huh7 cells are poor producers of IFN due to the lack of TLR3, worsening with each passage, and Huh7.5 cells further lack RIG-I perhaps increasing viral efficiency (77, 84, 85, 87-91). This view has come to change with the development of new liver cell lines (including one developed by our group named LH86 as well as immortalized non cancerous liver cells) highlighting the initial interaction of the virus and its probable role in the induction of IFN and its derived ISGs (77, 80, 82, 85, 92, 93). These new cells, combined with studies in primary hepatocytes and animal models, will help illustrate the initial interaction of HCV with the liver and demonstrate the dynamic equilibrium that can lead to viral escape or clearance.

1.4 Interferon Cascade

1.4.1 Early Events of the Type I IFN Response

In order to understand HCV's initial interaction with the host we first need to understand the IFN pathway. Most of the initial immune response is done through the recognition of the

virus' genetic material by pathogen recognition receptors (PRRs), such as TLRs and cytosolic receptors like the DexH(D) RNA helicase, retinoic acid inducible gene-I (RIG-I), followed by the induction of the type I IFN responses (88, 94-103). This is an intrinsic system in all cells and constitutes the individual source of immunity against invading pathogens. The IFN cascade starts with binding to specific sites inside the receptor, such as the leucine-rich repeat motifs in the ectodomain of TLR3 or the helicase/ATPase domain of RIG-I (104-108). This interaction leads to the binding of adaptor molecules specific for each receptor initiating the cascade (TRIF and Cardif respectively) (101). These events converge at elements that are part of transcription factors in charge of genes related to the amplification of the IFN signal and initiate adaptive responses. They are IRF-3, NFκB and ATF2/c-jun (104).

IRF-3 is constitutively expressed in cells were it awaits activation of the IFN cascade after pathogen recognition. It is activated by phosphorylation of its C-terminus which promotes its dimerization to either like particles or IRF-7 (109-112). In general, this phosphorylation is mediated by members of the non-canonical IκB kinases: IKKε and TBK-1 (Tank binding kinase 1, also known as NAK for NF-κB activating kinase)(94, 104, 109, 113). This event leads to nuclear translocation and association with CBP/p300 histone acetyl-tranferases binding on the DNA which transactivate the downstream genes of the early IFN response: IRF-7 (except on pDCs), IFNβ, IFNα1 and RANTES (114). HCV's non-structural protein NS3/4A has been demonstrated to block IRF-3's activation (NS3 serine protease/helicase domain by itself is not enough) with subsequent redistribution from the cytoplasm to the nucleus (84, 85, 88, 101, 104). These kinases do not seem to be affected by NS3/4A and furthermore, when overexpressed can affect viral replication (94). IKKε's main role, on the other hand, may be in the induction of HCV-dependent apoptosis due to its recruitment to the mitochondria (94, 109). A second IKK

complex is in charge of NFκB. This factor is normally repressed by IκB which gets removed by phosphorylation from the complex of IKKα, IKKβ and the regulatory subunit IKKγ (NEMO) (115). This happens by an interaction with Cardif in the mitochondria (which does not happen to TBK1) or TRIF in the cytosol (101, 109, 116). The end goal is the nuclear translocation of NFκB to initiate the transcription of pro-inflammatory genes. A third pathway is involved with the initiation of mitogen-activated protein kinases (MAPK) signaling cascades which leads to the activation of AP1 members. Some of these ISGs might also be induced by IRF-3 and NFκB without the induction of the IFN pathway (94).

1.4.2 Late Events in the Type I IFN response

Recognition of the invading virus, leads to a rapid cascade that ends with the induction of IFNβ and IFNα1. These then act in a paracrine fashion to continue on the enhancement of this innate response through the IFN receptor, formed from the dimerization of two components: IFNAR1 and IFNAR2. There is only one IFNβ but 11 subtypes of IFNα largely produced by immune cells whose particular purpose is not clearly understood (76). IFNβ itself can be subdivided into three pathways: 1) Insulin stimulation by IRS1 and 2; 2) MAP kinase and 3) ERK2 kinase (76). Later events in the response happen after IFN binds to the receptor just mentioned inducing a JAK/STAT signaling pathway which regulates the next step of the response by induction of ISGs including many pro-inflammatory cytokines (17, 76, 85, 94, 104, 109, 117, 118). HCV does not seem to directly interfere with this amplification part of the type I IFN pathway but disrupts the initial cascade involved in the production of IFNβ and the amplification loop (101). The end result is the reduced expression of IFNs and ISGs probably playing a role in the inefficient activation of the adaptive response to the virus.

1.4.3 Effects of the Cascade and Induction of Adaptive Immunity after HCV

One of the main consequences of the activation of innate immunity is the initiation of the adaptive response against the infection. In the case of HCV, a pro-inflammatory response is desired to induce viral clearance but it can also be the cause of pathology the leads to hepatitis. For instance, high levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα are induced and strongly correlated with liver damage in chronic HCV which is the reason why TLR agonists, which can induce a robust antiviral activity against the virus by producing them, are used with caution (17, 53, 64, 86, 119, 120). These cytokines may not act directly on the virus but they increase the levels of certain PRRs, like TLR3, inducing high IFN which maybe the reason why there is an increase in this response which favors viral clearance (118). As a matter of fact, the only cytokine that seems to have a direct effect against viral replication *in vitro* is IFNβ while the other cytokines have no direct effect this way (83, 86, 93, 121). They are induced by TRAF6 activated transcription factor called IRF-5 which is also responsible for IL-12, IL-18 and cyclo-oxygenase 2 (112). Interestingly, IL-12 is not induced in HCV but instead an increase in IL-10 is induced, which is better in terms of liver pathology, although at lower levels than IL-1β, IL-6 and TNFα (17, 86, 115, 122). Furthermore, the virus itself can mediate changes in their secretion to favor conditions for its propagation by inducing the secretion of IL-10 and TNFα by monocytes (Core and NS3)(17).

Other cytokines that are linked to liver injury are CXC and CC chemokines as they are also linked to the metastatic potential after transformation in chronic HCV (93). CCL3 is involved in hepatocyte inflammation (whose expression is increased in HCV infected patients and is linked with IFN non-responsiveness), CCL5 (also named regulated upon activation, normal T-cell expressed and secreted RANTES), CXCL8 (IL-8) and CXCL10 (IFNg activated protein IP-

10)(94, 123). Some of these chemokines are reduced by specific viral proteins because they are a direct consequence of the IFN pathway. For instance CCL5, CXCL10 and CXCL8 induced expression by Sendai virus-infection are reduced in response to full HCV genome or NS3/4A probably highlighting the role of PRRs in the induction of these chemokines (81, 84, 93, 101, 114, 123). In contrast, some of the viral proteins like the structural proteins or NS5A, can actually instead increase the levels of some or all of these cytokines although it may not be in an HCV RNA specific way (114, 123).

1.5 TLRs in the Recognition of HCV Genomes and Their Intermediates

To better understand the choice of pathway and the role it plays in HCV it is important to also understand the receptors themselves. Evidence suggests that the stage is set at the pathogen recognition level, more specifically TLRs which would correlate with a decrease in the type I IFN response. For instance, while they do not complete abrogate the defects observed, different TLR ligands are capable or overcoming some of them leading to DC maturation and activation of T_H cells in some cases (11, 49, 64). The livers of chimpanzees experimentally infected with HCV have a high induction of type I IFNs and ISGs even at the incubation stage which the virus evades and in human's co-infection with GBV-C seems to protect during co-infection due to the activation of the interferon system and the induction of maturation of DC (45, 124). Even more striking seems to be the fact that patients that have an increased expression of certain TLRs in their PBMC fared better in their response against the virus (64, 125). Conversely since most of these observations are clinical in nature they do not offer a point of view of the initial interaction with the virus. It also does not take into consideration the distinct subpopulations of DCs (described earlier) which can respond to pathogens in varied ways, even inside the same host. The two subtypes are characterized by different functions and receptors: for instance, mDCs and MoDCs express TLR3 and home to lymphoid organs while pDCs express mainly TLR7 and

TLR9 and are sometimes termed “professional interferon producers” due to their ability to produce close to 1000 times more type I interferon than any other immune cell (52). In the liver, while TLR7 remains restricted to pDCs, TLR3 is expressed not only by resident DCs and other immune cells but by hepatocytes as well (126).

Out of the 11 members of the TLR family, four are set for the recognition of foreign nucleic acid material. They are TLR3, TLR7, TLR8 and TLR9 all expressed by the liver (127, 128). TLR9 recognizes DNA material which is not part of the replicative cycle of HCV although it can be directly affected by HCV (38). TLR7 and 8 recognize guanosine or uridine-rich ssRNA but TLR8 does not induce IFNβ which is critical in inducing an antiviral state for this virus (102, 104, 129-131). They are also restricted in expression mostly to immune cells like pDCs. TLR3 recognizes double stranded RNA (dsRNA), including its synthetic analog polyinosinic–polycytidylic acid (polyI:C), by producing IFNβ and priming for an adaptive response (112, 115, 132). This last part can be achieved through cross-priming by conventional DCs which lead to the activation of cytotoxic lymphocytes (CTLs) to clear virally-infected cells (104, 112, 129, 133). These two receptors seem to be themselves focused on different subsets of cells for the induction of type I IFN since TLR3 may be present in the surface of endosomes of many cells including hepatocytes (requiring acidification for activation in DCs), while TLR7 seems restricted to the endosomal compartments of pDCs (88, 104, 112, 118, 134). It seems that TLR3 is ubiquitously expressed in many non-immune tissues which highlights its importance as a first line of defense against pathogens (132). Furthermore, both TLRs have been shown to induce a robust antiviral activity against HCV perhaps through structured dsRNA regions like 5’ and 3’ NTR (64, 81, 86, 94). TLR3 also can recognize HCV-infected apoptotic bodies it ingests by fusion with TLR-3 containing endosomes inducing its maturation (64, 73, 135). This function is

particularly important for the recruitment of NK cells and CTLs. Therefore, most of the focus of TLRs in HCV has been on the last two: TLR3 and TLR7, but mostly in immune cells.

Their role in liver cells or cell lines has not been deeply studied because of the use of hepatoma cells with defective IFN responses, as described earlier. Out of the functional model systems HepG2, HepaRG and LH86 cells have been shown to have active TLR3 and/or TLR7 pathways, not uncommon in tumor cells since these receptors may also be involved in tumor progression or apoptosis (92, 93, 120, 136). Immortalized human hepatocytes, like PH5CH8, also express TLR3 and further up-regulate it after poly (I:C) stimulation, just as cell lines with TLR expression do (80, 85, 118). Interestingly, in the case of chronically infected hepatocytes, the expression of these receptors has been shown to be down-regulated and to correlate with the dwindling levels of IFNα or with poor responses to IFN treatment in these patients (17, 120, 127). In this instance, it is likely the action of NS3/4A itself (which we will describe in particular later) interferes with the IFNβ promoter downregulating the expression of several key components of the cascade (84). During HCV infection complications (like glomerulonephritis, primary biliary cirrhosis or any type of liver inflammation) the virus seems to instead increase the expression of these receptors (118, 128). This disparity, and the actual role of the virus in it, requires further investigation. The *in vitro* correlation between IFNs, virus and PAMP expression could lead the way into a better understanding of what is happening in the chronically infected liver and the reasons why these pathways do not clear the virus *in vivo*.

1.5.1 HCV Directly Interferes with the TLR3 Pathway

TLR3 uses TIR-domain containing adaptor protein-inducing IFNβ (TRIF, also called TIR-domain-containing molecule 1 or TICAM1) while MyD88 is the adaptor for most other TLRs (101, 104, 112, 137). Signaling leads to the eventual activation of IRF-3 and NFκB (See figure 2

for a schematic representation of this pathway and HCV's effects on it). NS3/4A has been shown to directly cleave different adaptors for innate immune pathways with TRIF being one of them (between Cys-372 and Ser-373 which shares similarities with the NS4B/5A site on the HCV polyprotein) (64, 84, 88, 101, 104, 116). It was also demonstrated that the cleavage of TRIF impedes signaling by downregulating the levels of this adaptor and not due to any dominant negative activity by either section of the product (84). Interestingly, this was not observed in non-neoplastic cells PH5CH8 although it is not discounted that a balance in the levels of TRIF and the abundance of NS3/4A might play a role (64). In that light it is interesting to note that several HCV proteins (like envelope, core and NS5B) tend to induce IFNβ which may or may not get downregulated by overexpression of NS3/4A (80, 101).

After TRIF, the signal can be subdivided into four different pathways with different outcomes. For the production of type I IFN the signal goes to either TBK1/IKKε, or to PI3K which leads also to phosphorilation IRF-3 and IRF-7 making a heterodimer that induces IFNα (101, 104, 110, 138-146). PI3K-Akt pathway expression (below TLR3-TRIF) is not able to specifically induce IFNβ promoter activity after dsRNA stimulation (101). For the induction of NFκB, the cascade can go to either TNFR-associated factor-6 (TRAF6) or to Receptor Interacting Protein-1 (RIP-1)(104, 137). In chronic HCV patients the levels of IKKs expression are downregulated (94). Interestingly, over-expression of any of the IKK molecules before *in vitro* HCV infection seems to restore parts of the immune response to the virus and prevent its replication indicating that IKKs can inhibit even in the absence of IFN. This may be because NS3/4A does not seem to proteolitically cleave neither TBK1 nor IKKε, and even enhancement of their expression (by IFNα or TNFα addition to the media) fails to rescue the suppression in non-replicon cells (64, 84, 94, 101). As for RIP-1, it seems its role in HCV is not thoroughly

understood yet since it is related to viral induction of apoptosis, a new in HCV research, and because it is not completely involved in the IFN/HCV interplay which may not be the only anti-HCV pathway (81, 101). In this case, the induction of the IFN pathway induced by dsRNA made by the RdRp NS5B apparently from cellular DNA, without the need for replicating viral genomes (80). Furthermore, it delays cell cycle progression and decreases cell growth rates out of which TLR4 seems to play a higher role.

1.5.2 Case against TLR3

If TLR3 has the potential of being such an important PRR against HCV, what has kept it from being thoroughly studied? The answer comes from the reactions that this receptor causes in other infections. First, TLR3 is not a pre-requisite to responses to certain viruses and, second, in some cases lack of TLR3 reactivity leads to better outcomes such as is the case with West Nile Virus, Punta Toro hepatitis or with Influenza virus (104, 112, 115, 116). In both these instances a strong inflammatory response to clear the infection was the culprit for the early death of the host. Even our own group has previously demonstrated that decreasing pro-inflammatory responses while benefiting virus survival it also prevents some of the injury associated with the infection (147). Conversely, while injury is averted in these cases the viruses thrive and the potential remains for further disease. The case for pro-inflammatory responses in clearance is well known and highlights the need to get a balance between inflammation and clearance without permanent damage to the host. In that sense, it is understood that different viral stimuli can lead to different responses (112, 115). Therefore, a better understanding of the different combinations of responses against the viruses would greatly improve our understanding of how to clear HCV but how to approach other diseases as well.

1.5.3 Function of Other TLR and PRRs in HCV

TLR3 is not the only receptor recognizing HCV since cytosolic receptors RIG-I, PKR and 2^0-5^0 OAS have been shown to be involved in its recognition and are affected by the virus as well (further discussed later) (85, 148, 149). Interestingly, these pathways have been shown not to be redundant and it is thought that perhaps crosstalk between these pathways which may be the reason behind the varied modulatory effects observed after viral infection highlighted by their varied expression across cells and tissues (84, 101, 116). Futhermore, recognition of the viral genome, while being the most studied PAMP in HCV, does not preclude the recognition of other viral parts. In this case HCV proteins themselves can act as PAMP and induce IFN or modulate those responses (150). For instance, Core, NS3 and viral glycoproteins can interact and activate TLR2 or TLR4-mediated inflammation (64, 112, 128). The opposite has also been shown with NS5A were binding to the adaptor MyD88 (used by most TLRs except TLR3) and inhibit the recruitment of the kinase IRAK1 impairing the cytokine response (81, 128). It is important to fully understand not only how the viral RNA evades recognition but also how viral proteins can be recognized and may be engineered in the future to induce immunity against the virus during therapy.

1.6 Cytosolic Receptors in the Recognition of HCV Genomes and their Intermediates

1.6.1 RIG-I and the Cytosolic Recognition of HCV RNA

As described briefly in the previous sections, there are other PRRs involved in the recognition of cytoplasmic viral RNA (particularly uncapped 5'-triphosphate motifs as well as RNA composition) that can lead to the induction of type I IFN (64, 90, 104, 112, 115, 151-154). One of these is a member of a family of DexD/H box RNA helicases in the cytoplasm of cells named Retinoic Acid-Inducible Gene-I (RIG-I) which specifically binds to RNA secondary structures in the 5' or 3' NTR regions of HCV(see figure 3)(90, 104, 112, 116). Members of this

family can be induced by retinoic acid, IFNs and TNFα and are characterized by two-amino terminal caspase recruitment domains (CARD, critical for IFN induction) and a C-terminal helicase domain (101, 104). HCV is known to directly inhibit this pathway by the action of NS3/4A's protease activity on RIG-I (64, 145, 146). The importance of this receptor in the recognition of HCV is highlighted by the cell line Huh7.5 which contains a mutation in the first CARD domain of RIG-I. While binding of dsRNA to the receptor still occurs, downstream signaling is abolished by the mutation preventing the IFN induction. Furthermore, while not yet addressed *in vitro*, RIG-I has been shown to be downregulated in biopsy samples of chronic HCV patients (94, 104). This is believed to be the reason why is so permissive to this virus since lack of RIG-I correlates well with increased viral replication plus a mutation in its CARD domain allows replicon HCV replication in a non permissive cell (116). Interestingly, current studies have failed to increase permissiveness in these cells merely by restoring RIG-I or Cardiff over-expression indicating that more is at play (84, 88, 90, 104, 116). Furthemore, blocking IRF-3 did not increase replication efficiency of the virus in cell lines or in primary hepatocytes. The question remains as to the cause of such discrepancies: is it a technical problem such as a difference between stable transfection versus transient? Is it a reflection on viral mechanism such as the potency of the PAMP or the effectiveness of the cleavage of IPS-1 by NS3/4A? Is it due to the presence or absence of other receptors or even a crosstalk between them not yet studied? One answer may be in the sequestration of dsRNA from RIG-I by its negative regulator LPG-2 but only in minor terms since this protein is also downregulated in response to HCV (64, 90, 112, 152, 155, 156). Therefore, its real role in HCV remains unknown.

Just as with TLR3, the signaling cascade initiated by RIG-I leads to the activation of IRF3 and NF-κB leading to the induction of IFNβ (101). As a matter of fact, they both use the same

signaling molecules although the exact reason behind their different behaviors is not fully understood. After dsRNA binding RIG-I undergoes a conformational change that uncovers the binding site for its CARD domain which is part of what drives the signal transduction (90, 104, 116). This allows the association with itself and other molecules with the same domain on an adaptor protein that is mitochondria bound (90, 112, 116, 117). This molecule known as either Cardif/IPS-1/MAVS/VISA (from here on we will refer to is as IPS-1 since it seems to be the most commonly used) undergoes its own conformational change exposing the binding sites for the IKKs which get recruited to the mitochondria (101, 104, 116, 117). Similarly to TRIF, the carbocy-terminal region of IPS-1 can activate the IKKα/β/γ complex, IKKε and TBK-1 (although IPS-1 seems to prefer the first one) and is broadly expressed in tissues (94, 101, 104, 116). Out of these, the IFN pathway gets affected above IKKε since its overexpression can inhibit HCV in replicon cells (104). Evidence for the activation of this pathway on HCV comes from studying stimulated (with LPS, PMA or dsRNA treatment) or infected cells transcriptome which reveals the upregulation of genes that are normally directly induced by IRF-3 such as RIG-I itself, ISG15, ISG1-8, ISG56, ISG54, CXCL10, Viperin, NOXA, RANTES CXCL11, and USP18 (80, 94). Other molecules, such as TRAF6, TRAF2, RIP1, FADD and TRAF3, can also interact with IPS-1 through a proline-rich region at its N-terminus (104). RIG-I is in turn regulated by this cascade, specifically IRAK1 (122).

1.6.2 NS3/4A Activity against RIG-I

Upon entry, the HCV polyprotein gives way to molecules necessary to perform duties for the propagation of the virus. Some of these proteins can have pathogenic effects on the cells apart from their normal replicative functions. In the case of HCV non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B form a membrane bound complex for viral replication (91).

While some of them are actually attached to the membranes others are not or may localize to other places. NS3/4A, the protease/helicase complex, can localize also to the mitochondria where it serves a role in innate immune regulation (101, 117). NS2 can also inhibit the IFN promoter (although not as specifically as NS3/4A) eventually leading to the downregulation of different pro-inflammatory cytokines and chemokines like CCL5/RANTES and CXCL10/IP-10 but is not due to cleavage of IPS-1 (101). NS4B can instead induce the promoter activation although it has no viral functions currently ascribed to it and it does not colocalize with either the mitochondria or IPS-1 (123, 157). NS5A , a phosphoprotein, does not colocalize to the mitochondria with IPS-1 but with the ER membrane and inhibits the function of host antiviral proteins, for instance by binding to the kinase domain of PKR, by inhibiting IKKε expression in the mitochondria (although no change in activity) or by modulating cell-cycle regulatory genes (94, 101, 109, 123, 158, 159). It can also serve after NF-κB activation by shifting to the induction of IL-8 which would down-regulate ISG expression. Core (which partially colocalizes with IPS-1) and envelope proteins inhibit Jak-STAT pathway preventing the IFN amplification loops (109, 123, 160, 161). NS5B, the replicase, actually induces IFN probably by the production of dsRNA intermediates (even without the replication of the viral genome) but induces cell-cycle disruption by slowing the transition from S phase and the induction of IFN serves to make cells susceptible to DNA damage (80).

The protease NS3 itself can have more than one immunomodulatory function: Apart from inhibiting IFN downstream of both TRIF and IPS-1, it is also able to bind TBK1 and act as a competitive inhibitor of this kinase, also evidence by the fact that IKKε overexpression can restore IFN activation (101, 109, 112, 123). All of this leads to the conclusion that while most proteains have a role in immunomodulation, one of the most important viral factors in HCV is

NS3/4A since it has the most impact on the innate immune response. Furthermore, there is a demonstrated interplay between RIG-I and HCV's NS3/4A protease where RIG-I have been demonstrated to provide important anti-viral immunity while HCV has evolved ways to disrupt this response by interfering with both RIG-I and IPS-1 (112, 127, 145, 146, 162). This is part of the reason why this particular interaction is so studied since it can provide potential therapeutic targets against the virus (64, 163). Furthermore, RIG-I may have as of yet undetermined hepatic function since knockout of this receptor *in vivo* leads to fetal death by massive liver degeneration (104). Viral RNA is recognized by RIG-I which induces IRF3 activation but gets eventually overwhelmed after the protease levels manage to increase beyond a threshold (64, 101, 117). It is suggested that RIG-I might recognize RNA duplexes which form later than viral proteins including NS3/4A, this would be in accordance to RIG-I being overwhelmed by the protease before IFN levels reach sufficient levels to clear the virus or induce an effective adaptive response (85, 88, 109, 114). Similarly to other viral proteins, like 3ABC in HAV or NS3/4A in GBV-B, NS3/4A of HCV cleaves, in trans, within 5kDa of the short C-terminal transmembrane domain f IPS-1 at Cysteine 508 which dissociates it from the mitochondria and prevents downstream IRF-3 activation (85, 101, 104, 109, 112, 115-117, 123, 164). Consistent with this observation, the cleavage product of IPS-1 that moves from the mitochondria to the cytoplasm is also found on the cytoplasms of liver biopsies of chronic HCV patients (sera) and in the western blots of experimental cells (using genotype 1 or 2 HCV) (104, 112, 117). Furthermore, *in vitro* reproduction of these effects by siRNA silencing of IPS-1 mRNA not only prevents the IFN activation but leads to the enhancement of the HCV lifecycle in Huh7 cells and vice versa (88, 117). Conversely, other reports suggests that while RIG-I may play an important part in viral pathogenicity and development of chronicity, it might only be part of the story. First, the increase

of permissiveness of Huh7 or Huh6 cells after addition of a dominant negative RIG-I was only marginal and did not reach the replication levels by Huh 7.5 cells (88). Second, while the role of TLR3 in this interplay has not been well established, it is clear that NS3/4A disrupts this pathway as well and furthermore there maybe communication between both pathways that may be disrupted by the viral protease, further increasing cellular susceptibility (80, 81, 101). Third, the fact that NS3/4A interferes with RIG-I pathway does not indicate this as the pathway for development of chronicity since the proteases of acute viruses like GBV-B and HAV have a similar function (112). Fourth, IFNα or TNFα treatment cannot enhance the expression of molecules in the TLR3 and RIG-I pathway after suppression with NS3/4A and increasing the expression of IPS-1 in cells can only partially overcome it (101).

1.6.3 PKR and 2'-5'OAS as Separate Receptors and as Products of the IFN Pathway in HCV

PKR and OAS are induced in response to the invading pathogens by the IFN pathway as ISGs (89, 165, 166). Conversely, their presence can increment the recognition of viral genomes and in so doing helping amplify the response against it. Their role in HCV infection is not only denoted by their usual function but by the fact that NS5A and E1/E2 can directly bind either of these to prevent downstream signaling and amplification of proinflammatory responses (77, 82, 91, 123, 167-170). In the case of PKR (a serine/threonine kinase) this binding prevents its autophosphorilation and dimerization inhibiting the activation of initiation factor 2a (eIF2a) which aids in protein translation, halts proliferation and induces apoptosis although these have not been found to be conducive to the suppression of HCV (81, 91, 171-177). OAS's function on the other hand is as a ribonuclease that destroys viral dsRNA and also it serves more as an effector against HCV by inducing RNAse L destruction of dsRNA although it is also believed to play a minor role (115, 177). Both of these receptors are constitutively expressed in an inactive

form but their expression is upregulated by IFN. Conversely, a more prominent role for these cytosolic receptors should not be discarded as their role without NS3/4A evasion of TLR3 and RIG-I might be more prominent.

1.7 TLRs, RIG-I and the Induction of Cell Death

The idea of direct cytopathogenicity caused by HCV has lately come to light even though the induction of inflammation caused by infection in the liver. The main reason for this lack of insight was due to the fact that histologic examination of biopsies did not show any apoptosis or necrosis and because the majority of chronic carriers are asymptomatic (178-182). It is now understood that virus immune evasion maybe one of the reasons that not over cell death is observed since those samples are from patients were infection has formally established itself in the host and does not reflect an acute scenario (84, 109). In our case, the development of the cell line LH86 lead to the understanding that HCV may induce cell death in liver cells which does not happen in most other cells because they are selected for virus permissivity (92). Huh 7.5.1 cells were later also shown to have some level of apoptosis *in vitro* after virus infection although not extensive (73). The apoptotic bodies were capable of inducing a pro-inflammatory state by the induction of cytokines and the maturation of cocultured MoDCs. Even more interesting, is current non-HCV research that has layed the foundation to understand how the virus evades this branch of the innate response.

Recently it was discovered that death-domain containing RIP-1, which is normally associated with apoptosis by functioning downstream of TNFR by TRADD (a member of the TNF superfamily) interaction, can also associate with the TLR3 adaptor TRIF modulating the type of response (118, 128, 136, 137, 183, 184). RIG-I also requires TRADD for downstream signaling and induces NOXA and ApoL6 (proapoptotic genes regulated by the IRF3, IKKs and IPS-1) indicating a role in the development of cell death of the infected cell (94, 112, 183). This

would indicate that as long as both pathways are active (before evasion) active type I IFNs, pro-inflammatory cytokines and apoptosis can come together to induce viral clearance. Once the virus interferes with these two pathways the production or the synergy between these three effects disappears and maybe why in chronic infection cell death is not observed.

The question now remains, does the virus induce apoptosis by itself or is it a direct consequence of pathogen recognition? Certain groups have suggested the proapoptotic value of HCV proteins (like core or NS3) but it is debated wether this is a direct action or indirect due to the induction of IFN or pro-inflammatory cytokines (77, 185-188). More importantly, some of these proteins can use apoptosis as a way to reduce the number of immune cells like DCs which could also be detrimental to the hosts (189). Conversely, other groups have actually seen direct anti-apoptotic effects by proteins like NS2 and NS5A probably as an evasion mechanism by the virus (77, 101, 190-192). Perhaps cell death is not the cause of the inflammation as evidenced by the fact that TRADD-deficient mice can develop TNF induced hepatitis (183). One thing remains clear: there is a need to better understand the link between apoptosis and inflammation in order to gain a better understanding of how the virus balances recognition and evasion inside the host.

1.8 Concluding Remarks

It is increasingly clear that HCV is able to be recognized by the host cells and that furthermore that the liver itself holds the key to its own cure. While many questions remain unresolved, new information is linking the gaps in the knowledge and providing us with tools to create therapies against chronic infection. Perhaps some of these lessons will even extrapolate into other viral chronic infection as much as HCV researchers learn from other maladies. In the case of HCV it is also clear that the infection has many faces instead of the “all-or-nothing” approach to how the virus interferes with the host. Data is leading the way to understanding a delicate balance between the host’s immunity and the virus’ arsenal (see figure 1.4).

A strong immunity, while it may be the cause of cell death and perhaps hepatitis, can be the key for viral clearance while a weak response can be easily quenched by the virus to induce the chronic state. How to induce the first without incurring in consequences for the host should be the focus of future research in the area of chronic hepatitis infection. Are there tools currently in use in other diseases that can be useful for shifting these balances? Do we need to come up with *de novo* solutions? Is it stopping one viral protein like NS3/4A the panacea or does the answer lie instead in one of the other proteins without our knowledge? The knowledge that the initial response to the virus is critical combined with preventative measures on the population can help stave off chronicity. Induction of immunity by transduced DCs, increasing PRR expression or activating alternative pathways can lead to an early disruption of the viral life cycle and promote clearance. Perhaps the answer is already here and all we need to do is understand it.

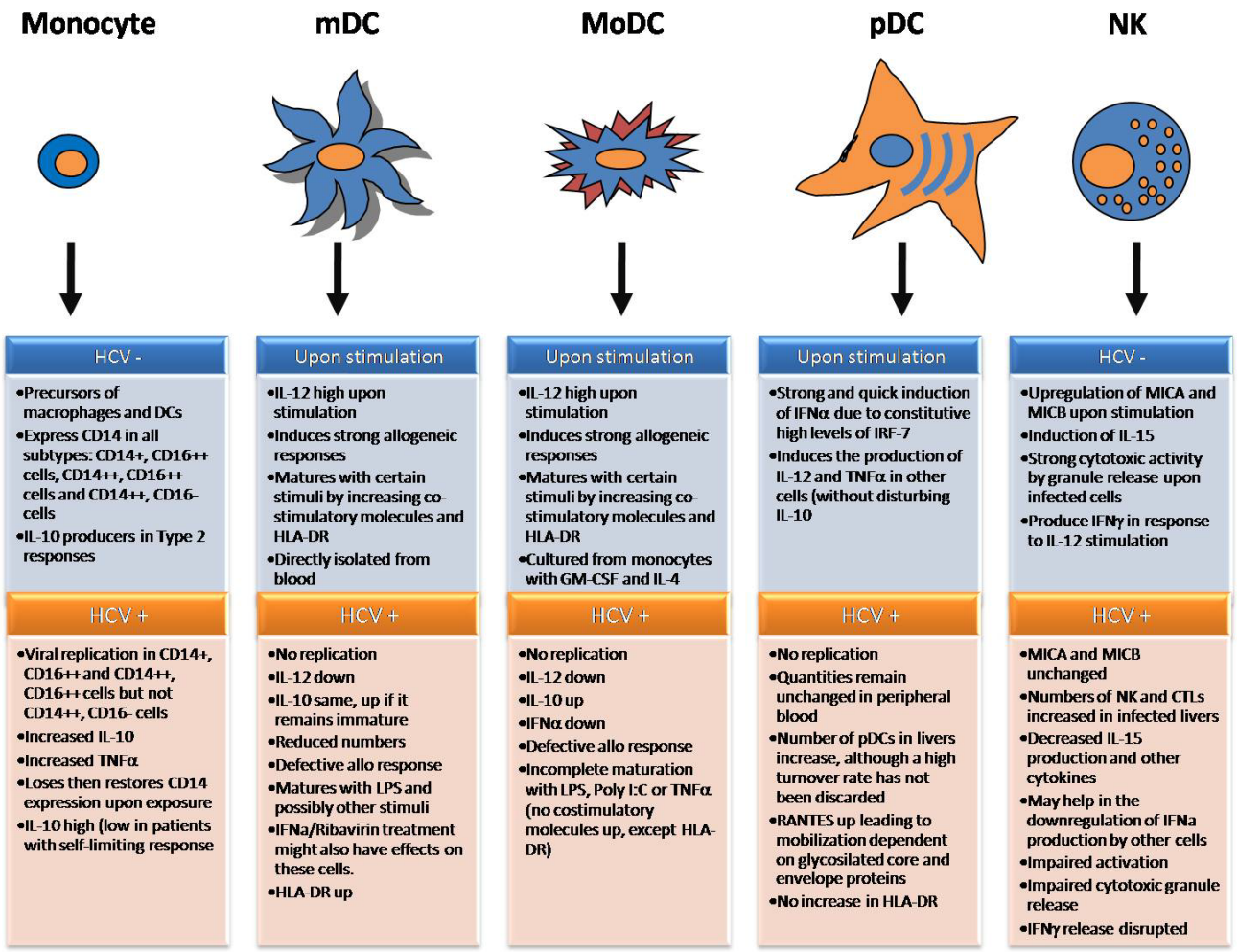


Figure 1-1. Comparison of the effects of HCV on DCs and related innate immune cells. The table represents a compendium of observations made either on clinical samples or cells from in vitro experiments. Each column is represented by a representative cartoon of each cell type

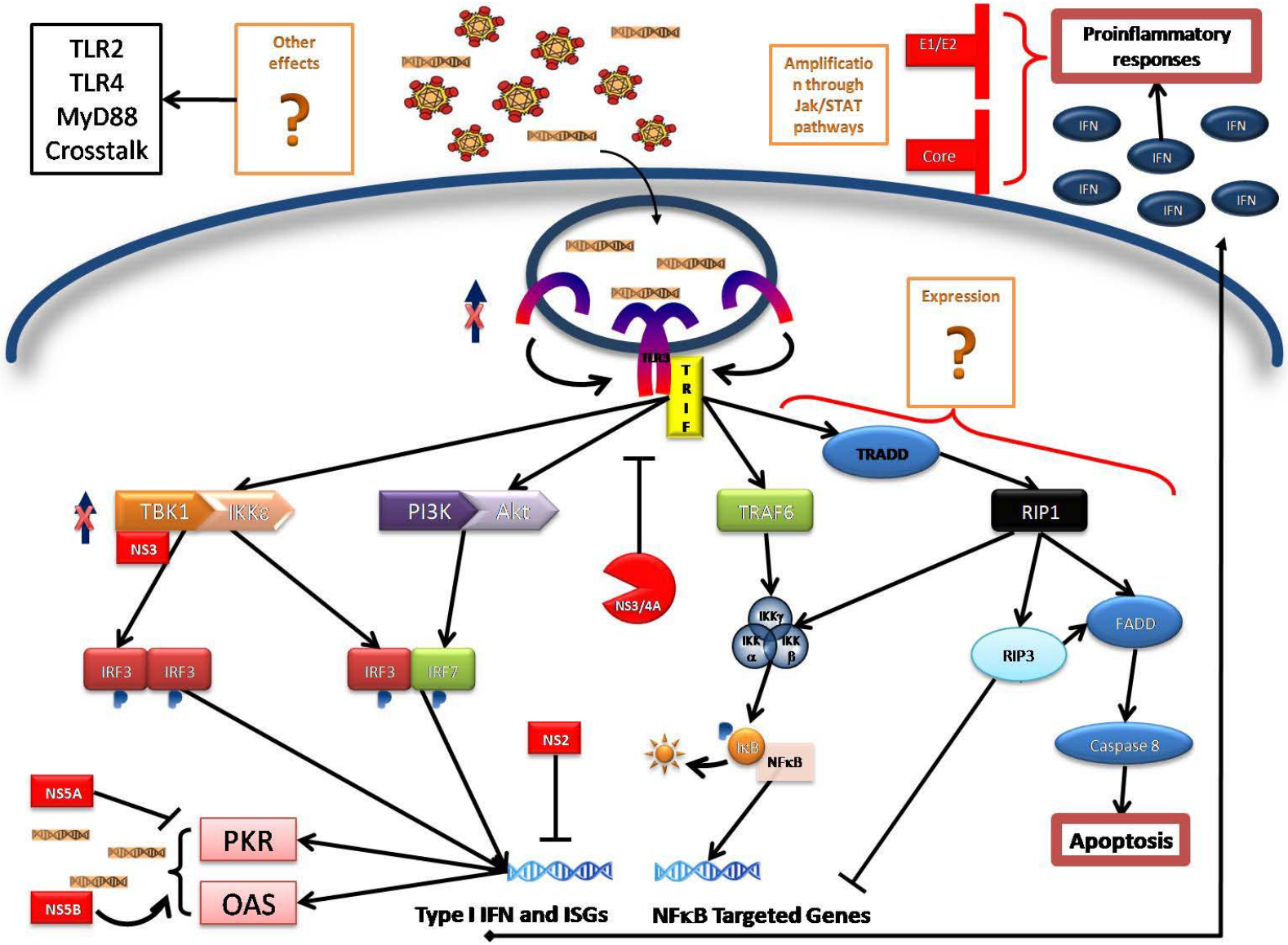


Figure 1-2. Schematic representation of HCV recognition and evasion by the TLR3 pathway.

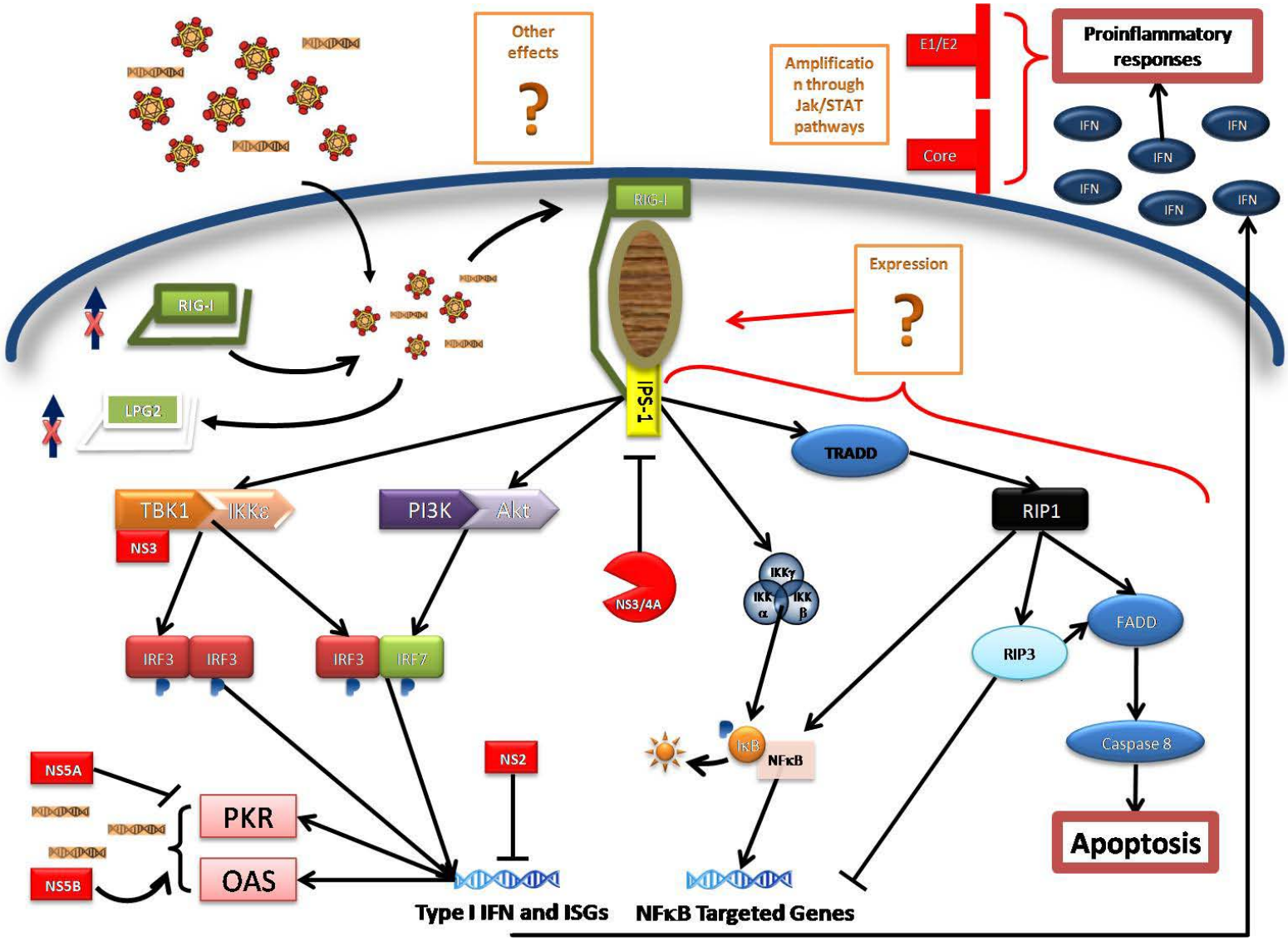


Figure 1-3. Schematic representation of HCV recognition and evasion by the RIG-I pathway.

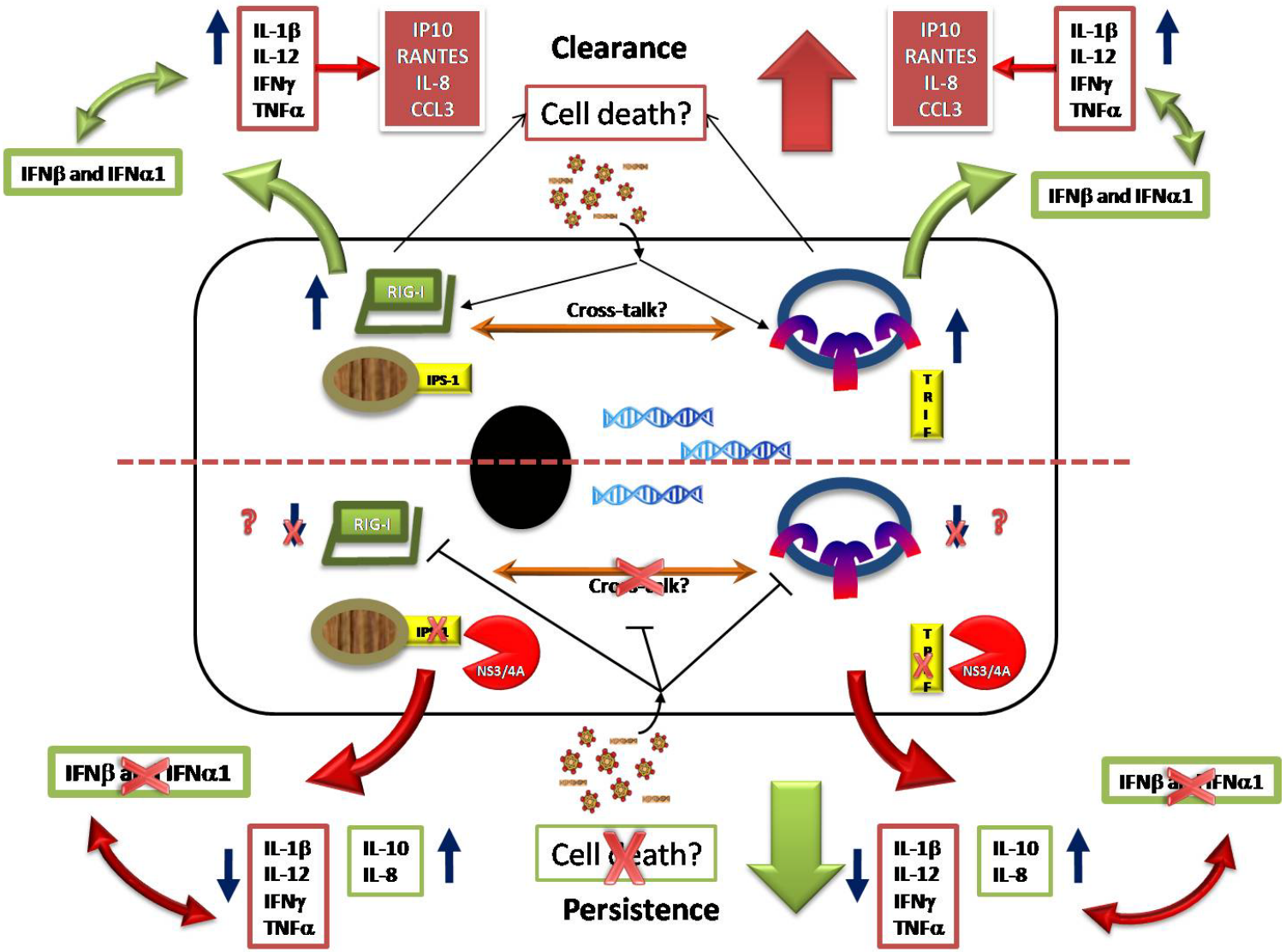


Figure 1-4. Model of acute interaction of hepatocytes with HCV.

CHAPTER 2
EXPERIMENTAL RATIONALE

Until now, it was challenging to experimentally study the virus and host cell interaction due to lack of cell models that support the whole viral life cycle. The cell culture system available in our laboratory, particularly the LH86 cell line we have established, has made this study possible. Our preliminary studies have suggested that virus undergoes changes for the species' survival benefit. We have found that HCV induces IFN production and apoptosis in acute infection, and this ability is lost in persistent viral infection. Our proposed experiments systematically examined the molecular mechanisms. The outcome of this study is important, because it will provide a scientific basis for novel HCV therapies by altering the hepatocyte innate immunity.

2.1 Hypothesis and Specific Aims

The main objective of this proposal was to investigate the intracellular innate immunity against HCV infection and how the virus escapes these defenses. HCV is a single-stranded RNA virus, which causes chronic infection in up to 80% of patients. The fundamental question is how the virus establishes a persistent and productive infection in the host when there is no generalized immune deficiency, as in most patients with this disease. A logical hypothesis is that HCV has developed strategies to evade immune attack.

Recent studies indeed shed some light on this aspect. However, how the virus escapes the first line of innate immune defense is not known. We developed, in Dr. Liu's laboratory, an infectious HCV cell culture system based on a novel liver tumor cell line (LH86). This system provides a unique opportunity for uncovering the interactions between intracellular antiviral defense and HCV infection. We found: 1) during acute viral infection (i.e., initial viral infection to host cells), the intracellular innate immune defense is activated through the production of type

I IFN. This is the first experimental model showing HCV can induce IFN in host hepatocytes; 2) HCV evolves into mutant viruses that do not trigger innate immune defense. The goal of my research was to examine how the cellular innate immune system reacts to HCV and how the virus develops strategies to evade this defense. I used the unique experimental systems established in our laboratory, including cell cultures that support the full life cycle of HCV infection, wild-type and mutant infectious HCV particles, and the experimental tools to study Toll-like receptor 3 (TLR-3), interferon stimulatory factor 3 (IRF-3), and other IFN signaling pathways. My proposal represents a novel and important initiative to study the interface between HCV and host cells. The overall goal was achieved through two Specific Aims:

Aim 1:To determine the effect of a direct HCV infection on antigen presenting cells. While there may be a specific role for these cells in the modulation of the adaptive immune response to the virus, most of the conclusions have been drawn from clinical samples or anecdotal data from patients. Our lab has the fully replicative HCV virus which was used to study the interaction of it with dendritic cells (DCs) at different stages of differentiation to observe effects on their maturation or immunomodulation. The importance of these cells stems not only from their important role in the initiation of adaptive response but also because of their impending use as therapeutics against many diseases, including HCV.

Aim 2: To determine how HCV induces type I IFN production in virus-infected human liver cells. The data shows that the HCV JFH-1 strain induces type I IFN production in LH86 cells, a new human hepatoma cell line established in our laboratory. This is so far the only cell culture model that produces IFN upon HCV infection. Using this model, we examined the role of TLR-3 and retinoic acid-inducible gene-I (RIG-I) in the induction of IFN. We also examined the viral components, RNA or proteins that are responsible for cell stimulation. The results will

provide novel insights into hepatocyte innate immunity in the overall intracellular antiviral network.

Our investigation aims at the interface between HCV and the innate immunity of target host cells. We believe this interface is fundamentally important to determine the fate of HCV infection. Understanding this interaction will provide critical knowledge of HCV-induced pathogenesis. It may be also instrumental for other viral infections. The outcome of this research will elucidate the role of intracellular innate antiviral immunity in the co-evolution of HCV and host cells. If our central hypothesis is correct, we should be able to test novel approaches to shift the balance between HCV and hepatocytes in the setting of chronic infection toward enhanced hepatocyte innate immune defense, resulting in elimination of HCV from the human host.

2.2 Originating Studies

Our preliminary studies demonstrated the feasibility for each specific aim and formed the foundation for the studies described in subsequent chapters.

2.2.1 The Signaling Pathways Responsible for Type I IFN Induction in Human Liver Cells

To examine the basic characteristics of type I IFN production in human hepatoma cells, we used Poly I:C to stimulate the cells. In contrast to many other cell types, human hepatoma cells do not respond to poly I:C in the culture medium, but only when Poly I:C was transfected into the cells by lipofectin (Figure 2-1).

Examination of Huh-7 cells indicated minimal TLR-3 expression. The proposal examine the role of TLR3. We therefore reconstituted the TLR3 in Huh-7 and its derived cell line, Huh 7.5, by plasmid-mediated over-expression. We confirmed the TLR-3 expression on these cells by western blot (Figure 2-2). These transfected cells were then infected with HCV-JFH1 viral particles and analyzed the IFNβ levels by Real Time RT-PCR. The presence of TLR-3 contributed to the induction of IFNβ in Huh-7.5 cells as compared to cells transfected with a

control plasmid (Figure 2-3). This shows the importance of studying the connection between TLR3 and the induction of type I IFN responses of liver cells in the context of a viral infection like HCV.

To examine the signal pathways that are involved in IFN induction, we transfected Poly I:C in Huh7 cells, followed by immunofluorescence staining of IRF-3. IRF-3 nuclear translocation was readily detectable, indicating its role in IFN induction (Figure 2-4). I conducted experiments to systemically examine the role of IRF-3 in LH86 cells.

To determine the pathways involved in IRF-3 activation, we employed siRNAs generated by T7 polymerase *in vitro* using the Ambion Silencer siRNA Construction Kit to downregulate the putative factors inside the cells. The construction began with PKR and RIG-I, as blocking IRF-3 completely eliminated IFN production, and blocking PKR and RIG-I partially eliminated IFN induction. Figure 6 also illustrates the efficiency of the depletion of the RIG-I gene with this method as it relates to the IFNβ response measured. I will conduct experiments in the proposal to systemically examine the role of IRF-3 in IFN induction in the LH86 cells with this method two DNA oligonucleotides (sense and anti-sense) synthesized by SigmaGenesis (St. Louis, MO). The efficacy of the target protein depletion was monitored by Western blot analysis. As shown in figures 2-5 and 2-6, the type I IFN production in hepatocytes appears to be dependent on IRF-3, and partially dependent on PKR.

2.2.2 Infectious HCV Cell Culture System

In 2005, several groups reported the successful culture of complete HCV (44, 193). The virus was originally derived from a fulminant hepatitis C patient, and it is genotype-2a. Intact and infectious viral particles were released after viral RNA transfection of the host cells (i.e. Huh7-derived). We have obtained the Huh7.5 cells from Dr. Charles Rice and the viral construct

pJFH1 from Dr. T. Wakita. Using a similar approach, we demonstrated efficient viral replication and production in this cell culture system. Almost 100% of the cells can be infected when released virus was added to Huh7.5 cells (Figure 2-7). Our monoclonal antibody against NS5A is reactive to this genotype 2a virus. The viral replication is relatively stable in this cell culture system, although fluctuation has been noted. We have been able to maintain the cells *in vitro* for more than 8 months. Supernatant from the cell culture contains abundant infectious viral particles (i.e.10^5-10^6 foci formation unit--ffu/mL), as determined by immunostaining after viral infection to a fresh cell culture. This culture system provides a critical source for infectious viral particles

2.2.3 Establishment of the LH86 Cell Line that is Susceptible to HCV Infection

Dr. Liu's laboratory has a program to culture primary hepatocytes and liver cancer cells from liver resections performed at UF-Shands hospital. One hepatoma cell line, referred to as LH86, was derived from a patient, who had no history of chronic liver diseases or viral infection. The tumor is well-differentiated, and the tumor cells exhibit enhanced epidermal growth factor receptor (EGFR) expression, as determined by immunohistochemical staining. The cells have a doubling time of approximately 42 hours in the presence of 10 ng/mL EGF (i.e. Huh7 cells doubling time 20 hours, Figure 2-8). The cells produce abundant human albumin and alpha-1 antitrypsin (liver-specific gene products). It appears that the cell line represents a well differentiated hepatocellular carcinoma. The derived cell line, LH86, shows a different morphology in comparison with the currently existent cell lines, such as Huh7 and HepG2. The cells form thickened cell cords (more than three-cell layers) resembling thickened liver plates in the original tumor tissue. These cells are also larger and have more cytoplasm that Huh 7.5 cells, indicating that LH86 cells are well differentiated tumor cells. We then tested the susceptibility of this cell line to HCV-JFH1 released from Huh7.5 cells. As shown in Figure 2-9, the LH86 cells

can be infected by the virus. These and other results that do the original characterization of this cell line have been already published (92).

2.2.5 HCV Induces Type I IFN Production in LH86 Cells

We know that HCV does not induce IFN in Huh7.5 cells. We next decided to examine whether HCV induces IFN in LH86 cells. To our surprise, infection of LH86 cells induced a robust IFN production (Figure 2-10 and (92)). This IFN response was only on replicating virus since denatured virus (by heat), virus treated with ultraviolet light (to prevent replication while leaving viral particles intact) or attenuation of virus (by multiple passages in culture) abrogated this effect This is so far the only human liver cells that can produce IFN in response to HCV infection.

We then examined the signaling pathways involved in IFN induction under this new system. IRF-3 is an essential, latent, transcription factor responsible for IFN induction. Upon activation, IRF-3 gets phosphorylated and forms dimers. Thus, the dimeric form of IRF-3 indicates its activation. As Shown in Figure 2-11, HCV infection induces IRF-3 dimers, and the formation of dimers is correlated with IFN induction. Furthermore, we also show that IRF-3 gets translocated to the nucleus of LH86 cells upon viral infection.

This correlates with the data on Huh 7 cells upon transduction with Poly I:C which correlated IRF-3 nuclear localization with IFN induction. We also examined the presence of TLR-3 in LH86 cells. Its presence in LH86 cells and not in Huh 7.5 cells also correlates with our IFN response findings in figure 4 that show that adding TLR 3 to Huh7.5 cells returned the IFN production in this otherwise unresponsive cell line (Figure 2-12).

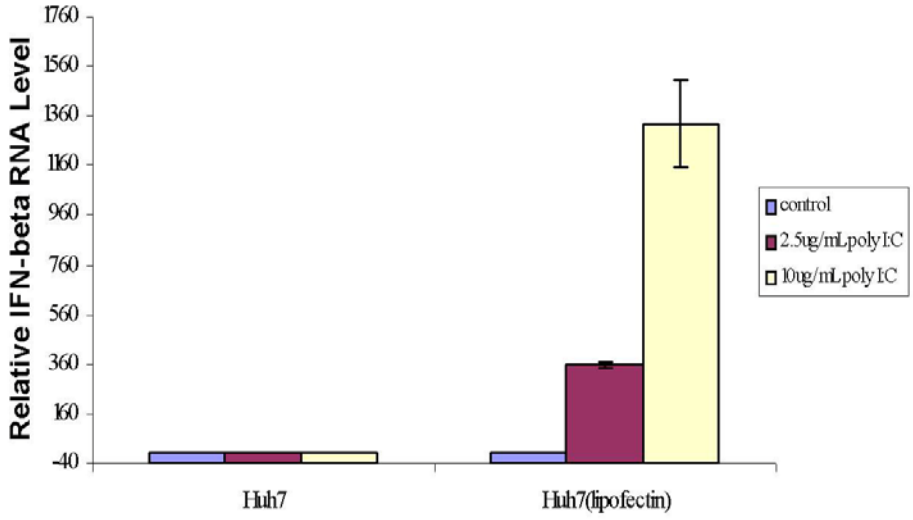


Figure 2-1. Type I IFN mRNA production is induced by intracellular poly I:C treatment in hepatocytes, while extracellular poly I:C cannot induce the expression of Type I IFN in hepatocytes. Huh7 cells were treated with poly I:C (with or without Lipofectin transfection) for 18 hours, followed by total cellular RNA isolation and real-time RT-PCR analysis of IFNβ.

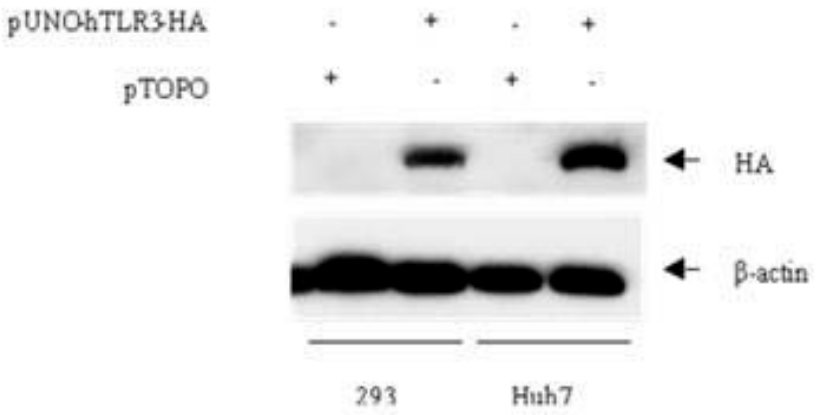


Figure 2-2. Expression of TLR3 in Huh7 cells upon transfection. Either pUNO-hTLR3-HA or pTOPO were transfected into Huh7 or Huh 7.5 cell lines. Protein was purified and analyzed using Western blot analysis with an anti-HA antibody.

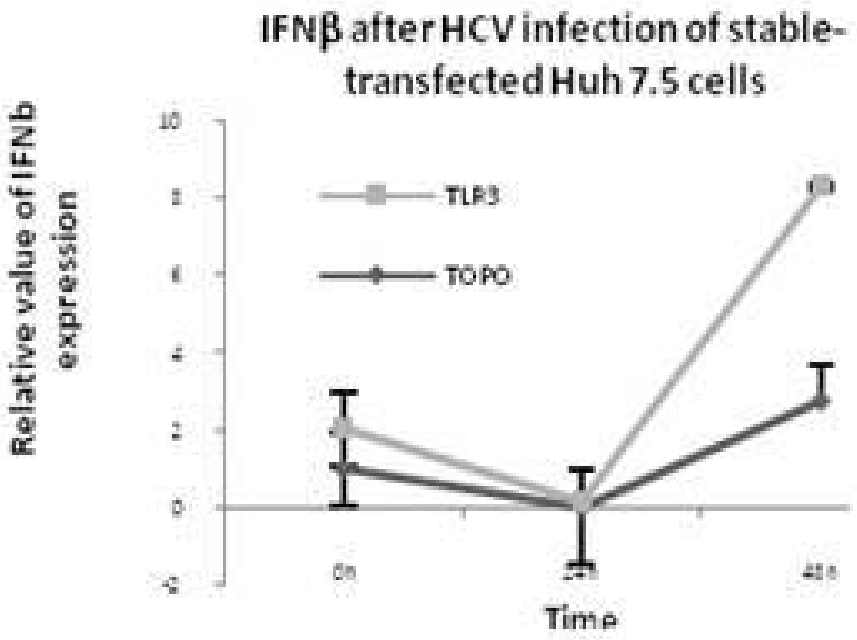


Figure 2-3. IFNβ is produced by Huh-7.5 that express TLR3. Huh 7.5 transfected with hTLR3 plasmid with lipofectin followed by multiple rounds of blasticidin selection were infected with HCV-JFH viral particles and the IFNb response measured by Real Time RT-PCR.

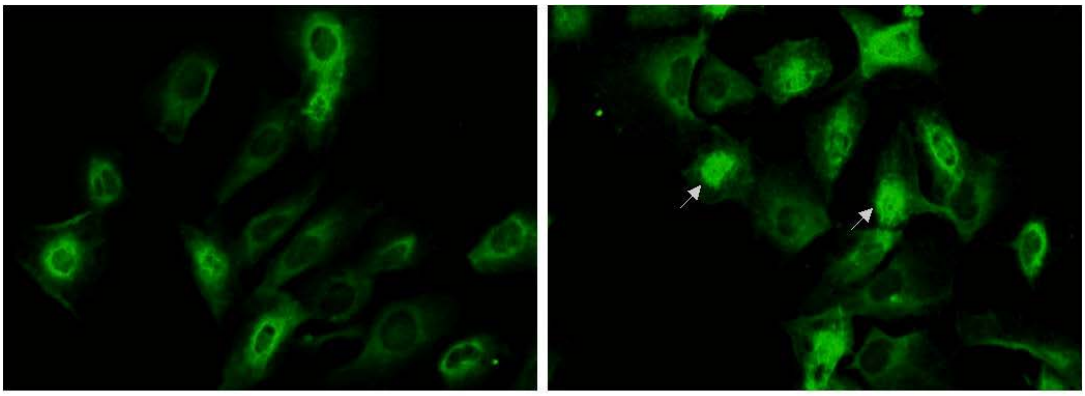


Figure 2-4. Nuclear translocation of IRF-3 in Huh7 cells. Huh7 cells were transfected with 2.5 ug/mL poly I:C for 24 hours, followed by immunofluorescence staining using anti-IRF-3 antibody. Arrows indicate IRF-3 staining in nucleus.

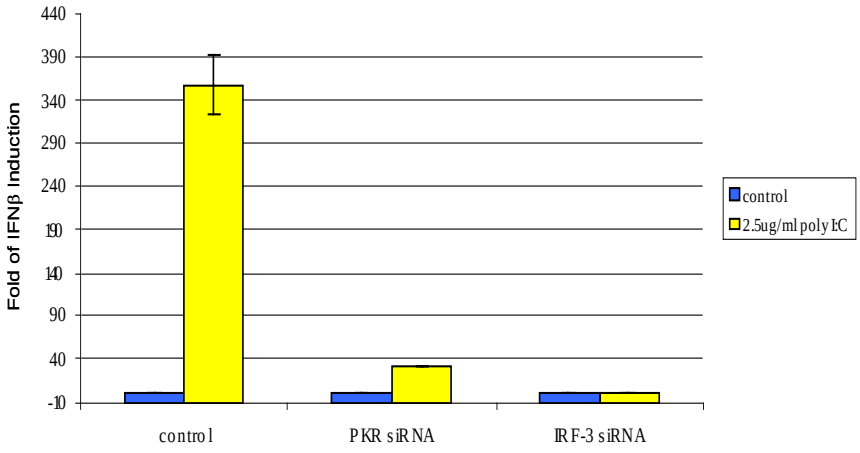


Figure 2-5. Type I IFN induction in human hepatoma cells. Huh7 cells were first transfected with control GADPH siRNA, PKR siRNA or IRF-3 siRNA for 24 hours, and then were transfected with poly I:C. After 12 hours, real-time RT-PCR was performed to assay IFNβ levels. The graph represents normalized data with non-polyI:C-treated cells as baseline level.

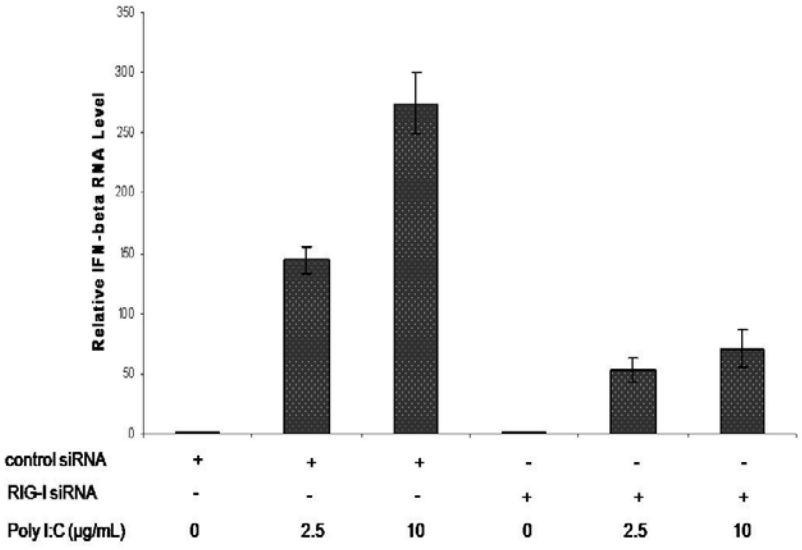


Figure 2-6. Production of IFN-β mRNA in response to dsRNA is partially dependent upon RIG-I in hepatocytes. Huh7 cells were treated with 2ug/mL of either control siRNA or RIG-I siRNA for 24 hours, followed by poly I:C transfection for 18 hours. Total RNA was isolated and IFN-β mRNA was analyzed using real-time RT-PCR, and IFN-β mRNA levels were normalized with GAPDH levels. Data are represented in triplicate.

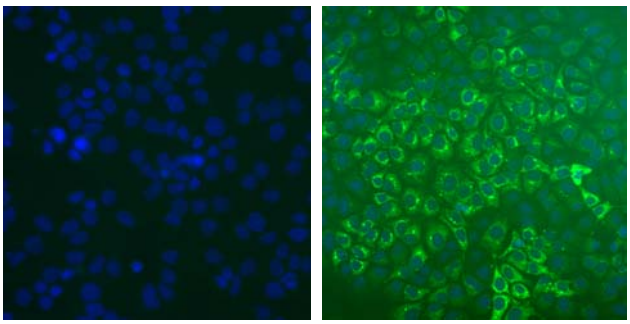


Figure 2-7. HCV-JFH1 virus is infectious in Huh7.5 cells. Immunostaining with anti-HCV NS5A antibody. The cells were harvested at day 7 after infection. The nuclei were stained by DAPI.

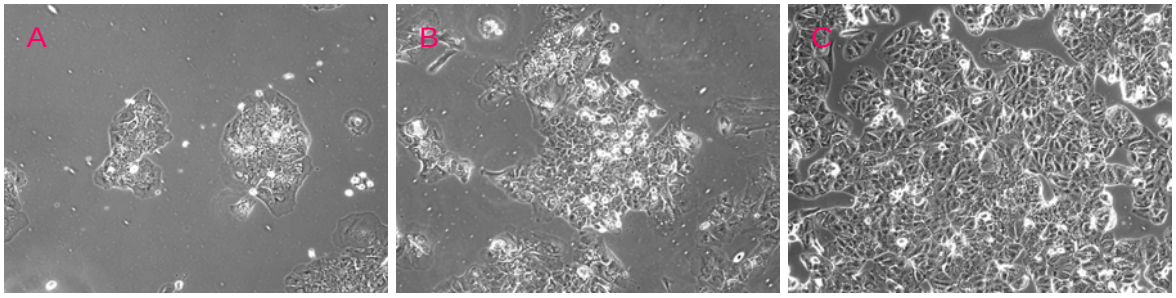


Figure 2-8. Morphology of LH86 cells in culture. LH86 cell morphology at different cell density. Photographs were obtained from an inverted microscope. A, B, C, represent different cell density on the culture dishes

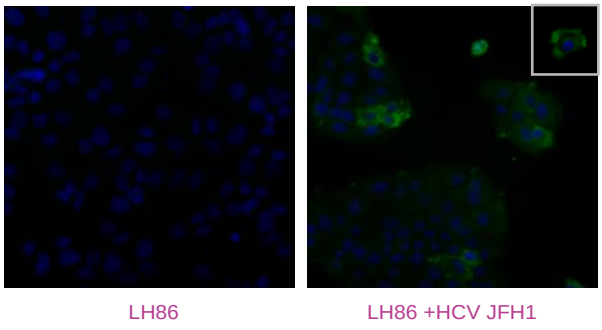


Figure 2-9. LH86 cells are susceptible to HCV infection. Immunofluorescence staining with anti-HCV NS5A antibody. Cells were infected for 72 hours.

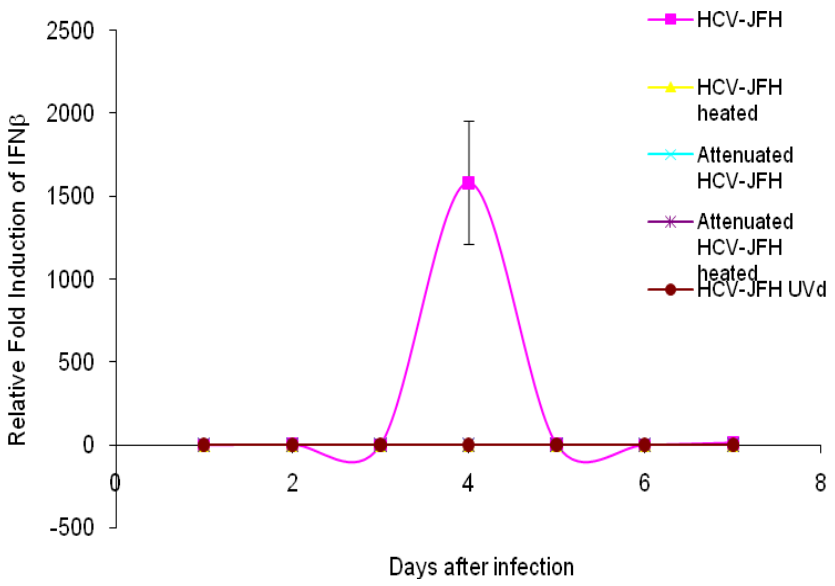


Figure 2-10. HCV-JFH1 virus induces IFN production in LH86 cells. The LH86 cells were infected by virus released from transfected Huh 7.5 cells. At the time points indicated, cells were harvested for RNA extraction, followed by real-time RT-PCR analysis with IFNβ primers. The data were normalized with internal control GADPH.

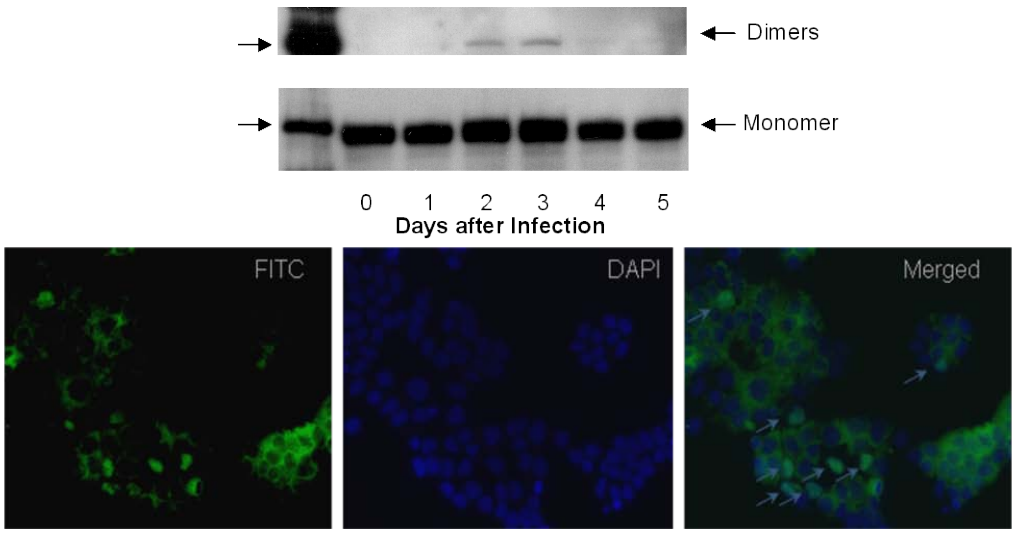


Figure 2-11. HCV-JFH1 virus activates IRF-3 in LH86 cells. The LH86 cells were infected by virus released from transfected cells. At the time points indicated, cells were harvested for protein extraction, followed by Western blot analysis using anti-IRF-3 antibody. IRF-3 was also detected by immunoflorescence. Arrows indicate nuclear localization of IRF-3.

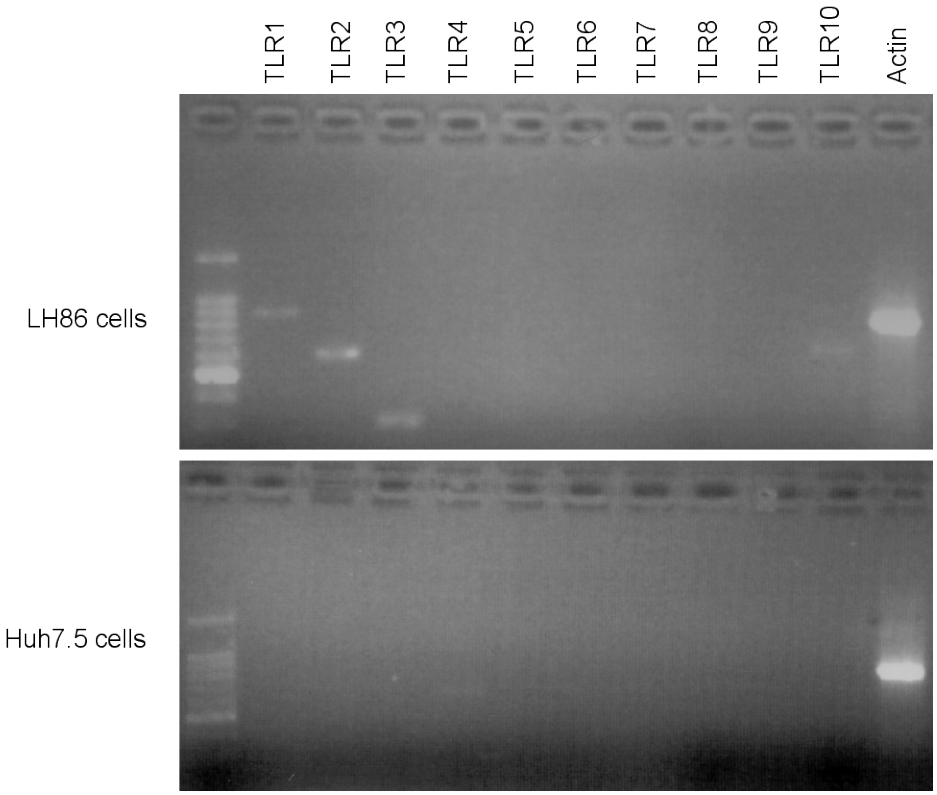


Figure 2-12. Expression of Toll-like receptors in LH86 and Huh7.5 cells. This quantitative RT-PCR assay shows the expression of TLR-1, 2, 3, and 10 expression in LH86 cells. There is only faint TLR4 expression in Huh7.5 cells. Actin serves as an internal control.

CHAPTER 3
HCV INTERFERES WITH THE INNATE RESPONSE OF HEALTHY HUMAN MONOCYTE-DERIVED DC

3.1 Materials and Methods

3.1.1 Isolation of PBMC and Culture of Monocytes and DC

Buffy coats (leukopac, PBL) were diluted three times its volume in 1X PBS pH 7.4 (Gibco, California, USA). The dilution was layered onto Lymphoprep (Axis-Shield, Norway) in a 2:1 ratio for Ficoll-Hypaque density gradient centrifugation and centrifuged for 25 minutes at 22◦C and 1200 rpm. PBMC were collected at the interface, washed twice and centrifuged each time for 10 minutes at 4◦C and 1200 rpm. Cell viability was assessed by trypan blue exclusion. All cultures of human PBMC and derived cells were maintained in RPMI 1640 medium (Sigma, Missouri, USA) supplemented with 2 mM L-glutamine (Life Technologies, Paisley, Scotland), 5000 U/ml penicillin (Sigma, Missouri, USA), 5000 U/ml streptomycin sulfate (Sigma, Missouri, USA), and 10% v/v fetal bovine serum (Gibco, California, USA) named cRPMI.

Monocytes were obtained by adhering 5x106 PBMC/well to a 6 well culture dish for 2 hours at 37oC. After aspirating the non-adherent cells, monocytes were washed with 1X PBS pH 7.4. Complete media was added to the remaining cells. To induce differentiation into DCs, monocytes were cultured with 50ng/ml GM-CSF (BD Biosciences) and 20ng/ml IL-4 (BD Biosciences) for 6 days. Maturation was induced with 1ug/ml LPS (Sigma) for 24 hours.

3.1.2 HCV Constructs and Viral Particle Generation

The linearized genomic pJFH-1 plasmid DNA was purified and used as a template for *in vitro* transcription using MEGAscript kit (Ambio, Autin, TX), delivered into Huh-7.5 cells by electroporation and cultured in cDMEM. Cells were passaged every 3-5 days and, at 21 days, supernatants were filtered and frozen. Viral titer ($1x10^5$ ffu/ml) was determined by the average number of NS5A-positive foci detected at the highest dilutions. Infection was done by adding

supernatant or supernatant diluted in cDMEM for 24 hours before exchange. Control is uninfected-Huh7.5 supernatant.

NDV strain La Sota was added to uninfected CHO cells cultured in cDMEM for 2 days. Cells and media were subsequently exposed to 3 cycles of freeze/thaw treatment (i.e., -80^{o}C and 32^{o}C). The supernatant was centrifuged at 12,000rpm for 10 minutes to remove cellular debris and then aliquoted and frozen at -80^{o}C until use.

3.1.3 Immuno-Fluorescence

Incubated DCs were cytospun onto a coverslip (Fisherbrand) and Huh 7.5 cells were grown on them then fixed with acetone on ice. After three washes with 1XPBS, cells were incubated with mouse anti-HCV NS5A monoclonal antibody (generated by Dr. Johnson Lau in the Hybridoma Core Laboratory at the University of Florida, clone number HL1126) for 1 hour followed by goat anti-mouse IgG-FITC (Southernbiotech, Birmingham, AL) for another hour. Cells were examined under a fluorescent microscope (Olympus).

3.1.4 Reverse Transcription and Real Time Polymerase Chain Reaction

Total cellular RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription to obtain cDNA was performed using the Superscript II (i.e.: 50 U reverse transcriptase per reaction) first-strand synthesis for RT-PCR kit (Invitrogen) primed with oligo (dT)(Invitrogen) according to the manufacturer's instructions. Quantitative real-time fluorophore-labeled LUX primers pairs were obtained from Invitrogen (table 1). The PCR conditions were: 50oC, 2'; 95oC, 2' (95oC, 15"; 60oC, 30" (IFNs,HCV) or 62oC, 30" (GAPDH); and 72oC, 1 min) for 45 cycles. Reactions were monitored at every cycle during the annealing step on a spectrofluorometric thermal cycler (MJ Research DNA Engine Opticon® 2 thermal cycler, BIORAD). Results were analyzed with MJ Opticon Monitor 3.1 software from BIORAD.

Table 3-1. D-Lux Primers used

5' UTR	5'-CGCAGGAGAGCCATAGTGGTCTG[FAM]G-3' 5'-GAGCGGGTTGATCCAAGAAAG-3'
IFNβ	5'-CAGCCGATTCATCGAGCACTCGC[FAM]G-3' 5'-TTCCAGGACTGTCTTCAGATGG-3'
IFNα2A	5'-GACTCCCTCACAGCCAGAAATGGAG[FAM]C-3' 5'-ATGACCTGGAAGCCTGTGTGat-3'
GADPH	5'-CGACCGGAGTCAACGGATTTGGT[JOE]G-3' 5'-GGCAACAATATCCAGTTTAGCA-3'

3.1.5 Flow Cytometry

DCs were assayed by four-color immunofluorescence staining with a panel of directly conjugated antibodies. One million cells were stained with lineage (Lin) fluorescein isothiocyanate (FITC)-conjugated antibody cocktail (CD3, CD14, CD16, CD19, CD20, CD56) and HLA-DR PerCP-conjugated antibody (BD Biosciences, Heidelberg, Germany). The presences of maturation markers (CD80, CD83, CD86 and CD40) as well as the HCV binding receptor, CD81, were also measured. Pelleted cells were incubated for 30 min at 4°C with antibodies, washed with staining buffer (PBS + 2% BSA + 0.1% Na azide), and then fixed with 2% paraformaldehyde and stored at 4 °C. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Quantitation was done by the CellQuest software (BD Biosciences; version 3.2.1). Isotype-specific immunoglobulin controls were run for each fluorochrome. Twenty five thousand cells were analyzed for each sample.

3.1.6 Enzyme-linked Immunosorbent Assay (ELISA)

After culture, aliquots of supernatants were frozen and stored at -20°C. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with 1 mg/ml of purified monoclonal antibody against human IL-12 (p70), IL-10, TNFα or IFNγ (Pierce-Endogen, Rockford, Illinois) in coating buffer (1X PBS, pH 7.4) at room temperature overnight. The plates

were then incubated in blocking buffer (1X PBS, pH 7.4 with 1% bovine serum albumin [BSA]; Sigma, Axel, The Netherlands) for 2 hours at room temperature and washed in 0.05% Tween-20. Standard serial dilutions were added to the plates as well as 50uL of supernatant in triplicate followed one hour later by biotin labeled anti-cytokine antibody. Recombinant human TNFα (hTNFα), hIFNγ, hIL-10, and hIL-12 p70 were used as standards in the assays, as provided by the manufacturer. Cytokines were captured by the specific primary monoclonal antibody and detected by biotin-labeled anti-IFNγ, anti-TNFα, anti-IL-10, or anti-IL-12 (Pierce-Endogen, Rockford, Illinois). The color reaction by strepavidin-horseradish peroxidase was incubated for 20 minutes and developed by adding TMB microwell peroxidase substrate (Pierce-Endogen) and stopped by the addition of an equal volume of 0.18M H_2SO_4. The absorbance was read at 450 nm.

3.1.7 Autologous Mixed Leukocyte Reaction

Autologous PBMC were resuspended at $5x10^6$/ml in sterile PBS and labeled for 15 minutes with 200nM CFSE according to manufacturer's protocol (Molecular Probes, Eugene OR). Washed cells were then seeded on a 96 well plate at $5x10^5$cells/well. Monocytes, immature DC or mature DC were added at the ratios 1:1, 1:10, 1:50 and 1:100. Positive control wells received lectin (PHA; 5ug/ml; Sigma, Missouri, USA) for non-specific stimulation of lymphocytes. Cells were cultured for 6 days at which time cells were harvested and stained for flow cytometry as described above for the following markers: CD3 APC, CD4 PerCP and CD8 PE.

3.2 Results

3.2.1 HCV Induces Phenotypical Differences in Monocytes and Immature DC but not on Mature DC

Since HCV has been reported to interfere with the function of DCs and these effects seem viral titer dependent, we decided to study the direct interaction of the virus on these cells. We cultured enriched monocytes, immature DCs or mature DC with either MOI of 0.1 or an MOI of 0.01 (Figure 3-1).

LPS was chosen to induce maturation as the first results demonstrated an impaired DC allostimulatory function in chronic HCV infection were obtained with DCs treated with LPS (28). Furthermore, recent data reported a significant role of LPS in the pathogenesis of HCV infection, suggesting that the maturation stimulus may have physiological importance *in vivo* (62). We were able to observe a shift in the monocyte morphology, by light microscopy, to a more adherent phenotype with many processes after culture with a high MOI of HCV compared to control (Figure 3-2 A and B). However, the morphology of monocytes after a low MOI of HCV did not change (Figure 3-2C). Immature DCs had a higher number of non-adherent clusters after addition of a high MOI of HCV but again not with low MOI (Figures 3-2 D through F). Mature DCs did not show any morphological differences after addition of virus (Figures 3-2 G through I).

To further characterize these observations we analyzed the cells by flow cytometry for the presence of DC (HLA-DR^+Lin^-). Monocytes are Lin^+ because of the presence of CD14 which is also present in macrophages. They showed a phenotypical change towards DCs by losing the lineage marker during the first 24 hours of culture with a high MOI, reverting back to a Lin^+ phenotype (might have returned to monocytes or become machrophages) after a week of culture, while a low MOI did not lose the marker but increased the amount of Lin^+ cells at the beginning

of the experiment (Figure 3-3 A through D). Immature DC percentages when cultured with HCV (either MOI) increased but only the low MOI treated cells reverted back to the original percentages after 7 days of culture (Figures 3-3 F through J). Mature cells showed no strong differences as compared to control (Figures 3-3 K through P). These correlated with the morphological characteristics observed under the microscope. These cells remained immature since they did not up-regulate co-stimulatory molecules (CD40, CD80, CD83 or CD86) at either MOI (data not shown).

3.2.2 HCV Affects the Proliferation of T Helper and CTLs at the Basal Level by Affecting DCs

In order to further understand our observations, we tested the ability of these cells to induce the proliferation of naïve T cell subsets in PBMC. We chose to study autologous cells since our observations indicated that HCV does not interfere with the maturation or activation of cells by other means, such as LPS, and because it is a way to really understand virus-specific responses (16). In the PBMC population we gated for T helper cells ($CD3^+CD4^+$, Figure 3-4 A through C) as well as the CTLs ($CD3^+CD8^+$, Figure 3-4 D through F).

As expected, the amount of proliferation of T helper cells on healthy PBMC did not change with increasing amounts of un-incubated monocytes while there was a slight increase in the basal PBMC proliferation in control immature and mature DCs that was dependent on the ratio of DCs added to the coculture. When monocytes or DCs were incubated there was some notable effects on T helper cells: 1) when co-cultured with monocytes there was an increase in proliferation but it was inversely correlated to the amount of virus; 2) there was a decrease in the amount of proliferation induced by co-culture with immature DCs; 3) contrary to immature DCs, there was an increase in the amount of proliferation induced by mature DC when they are incubated with HCV.

As for CTLs in the PBMC, they are not directly affected by either cell since an increase in the ratio of monocytes or DCs did not change their proliferation but cocultured with these cells when incubated did have an effect in their proliferation. Monocytes up-regulated only at a low MOI of 0.01, similar to what was observed with T helper cells. Conversely, incubated, immature DC co-culture strongly downregulated the basal proliferation of CTLs in the culture. Since they are not dependent on the ratio of cells it might be an indirect effect likely from the T helper cells since they share a similar pattern.

3.2.3 HCV Affects Type 1 Interferon Responses in the Absence of Replication

In order to see if the cell recognizes HCV we studied the effects of the virus on the type I interferon responses (i.e.: IFNα2a and IFNβ) in monocytes, immature DCs and mature DCs. Monocytes did not produce IFNβ when exposed to HCV (Figure 3-5 A).
Furthermore, HCV downregulated the basal levels of expression in these cells with longer exposure to HCV resulting in the lowest expression of INFβ. DCs were able to express IFNβ in response to HCV, while mature DCs producing tenfold more than immature DCs (Figure 3-5 B and C). In contrast, IFNα2a was produced consistently in the three groups. It was interesting to note that immature DCs had the highest expression of the three cell types (Figures 3-5 D through F).

These results indicate that HCV seems to downregulate IFNβ in monocytes and immature DCs. To understand this further, we tested the effects of this inhibition on the IFNβ produced by another virus. For this purpose we cultured monocytes and immature DC with Newcastle Disease Virus (NDV), HCV (either MOI) or both. Both monocytes and immature DCs were able to induce IFNβ in response to NDV with DC producing a much higher response than monocytes, as

anticipated (Figure 3-5 G and H). The presence of HCV significantly reduced the IFNβ produced in response to NDV.

We next measured the replication of HCV on all the cells by Real Time RT-PCR. As reported by others, HCV replicated in monocytes (194). Interestingly, DCs, (mature or immature) did not show signs of replication after seven days of culture (Figure 3-6 A and B). To further corroborate that there was no replication we did immunofluorescence staining against NS5A on DCs. This assay also did not reveal the presence of HCV in these cells but did on the positive control (Huh7.5 cells with pJFH1, Figure 3-6 C, D and E). The presence of CD81 was unchanged by the virus (data not shown).

3.2.4 HCV Interferes with IL-12 and IFNγ production in monocytes and DCs

A strong type 1 response has been heralded as an important step towards clearance of HCV infection in acute and chronic treated HCV patients. Therefore we decided to study the role of HCV in the production of IL-12 as a marker for the initiation of a T_H1 response. The supernatants of the experiments described in figure 4 were measured for IL-12. Monocytes did not have a reduced IL-12 production by day one with either concentration of virus. By day 7, MOI=0.1 did not show a significant difference while MOI=0.01 showed a decrease against the day 7 control monocytes as measured by paired student t-test (Figure 3-7A, $P<0.05$). Immature monocytes also did not show a significant change in the quantity of IL-12 by day 1 but, unlike monocytes, they produced less IL-12 with the higher titer of virus but significantly higher IL-12 with MOI=0.01 (Figure 3-7B).

Mature DCs were able to produce much higher amounts of cytokines in general as compared to the other two types of cells and they secreted significantly less IL-12 at any of the viral titers tested with MOI=0.01 being the lowest IL-12 between the two (Figure 3-7C). Since

IL-12 is important for induction of the pro-inflammatory cytokine IFNγ we tested it to corroborate the effects of HCV on IL-12. None of the three cell types produced IFNγ in response to HCV as compared to control although mature DCs were able to produce more than any of the other cells Figures (3-7D through F).

3.2.5 IL-10 and TNFα in Monocytes and DCs after HCV Infection

IL-10 and TNFα are two cytokines that have been correlated in the pathogenesis of HCV in the liver of infected patients. Therefore we decided to measure the levels of these cytokines. Monocytes infected with HCV were able to produce a significant increase in the levels of IL-10 by day 7. There also seem to be a dose dependency since the higher HCV MOI produced a higher amount of IL-10 (Figure 6A). In contrast, immature DCs were able to significantly decrease the levels of IL-10 produced after infection with the higher MOI from day 1 and by both MOIs by day 7. By day 7 the levels of IL-10 expressed were similar and different titers did not reflect differences (Figure 6B). Mature DC did not show differences at day one but showed a significant decrease in expression at the lower MOI which was not significant in the higher titer (Figure 6C). Therefore HCV has a very contrasting role in the acute production of IL-10 in DCs as compared to monocytes. The pro-apoptotic cytokine TNFα in monocytes or immature DCs had a tendency to decrease while mature DCs showed a significant dose dependent increase on day 1 with a significant decrease, only in the lower MOI, by day 7 (Figures 6D though F). Therefore we found no specific correlation between monocytes and DCs after infection with HCV and chronic liver disease.

3.3 Discussion

One of the main questions regarding the defect in the function of DCs in HCV infection is whether it is a direct consequence of viral infection. While there is direct evidence about the

replication of HCV in monocytes, the same cannot be said for DCs were detection of HCV genomes within isolated DCs has been inferred as indicative of replication (16, 28, 37, 195). Moreover, MoDCs responded to HCV with phenotypical and immunological changes, in the absence of replication, including the modulation of IFNβ. This phenomenon is corroborated by the results obtained when co-infecting with other virus. HCV impaired NDV-induced IFN production by DCs and monocytes indicating that HCV negatively regulates the induction of IFNβ by these cells in a way dependent on the amount of virus. Such an observation has also been made on chimpanzees, chronic and healthy patient cells were they saw an induction of type I IFNs and ISGs even in the incubation phase although lower on chronic patients (45). This correlates with data that shows the presence of HCV genomic RNA in MoDCs alters their immunostimulatory capacity and that the ability of DCs to stimulate T cells can be disrupted by downregulation of MHC I and co-stimulatory molecules, independent of replication in other viruses, which could suggest an initial mechanism that can lead to a chronic state (16, 36, 196). One explanation for this is that viral proteins can have immunomodulatory effects upon binding. Immature DCs for instance are capable of binding HCV-LPs in an envelope-specific, concentration dependent manner which induces DC activation (40, 41). One such binding mechanism suggested in the literature is the interaction of DC-SIGN with E2 which protects the virus from lysosomal degradation (16). Furthermore, several of the HCV proteins have been shown to directly affect the immune responses including the envelope and core proteins which have been shown to interfere with the type 1 IFN responses (170, 197). Wether this is the exact mechanisms of IFN downregulation even in the absence of replication is something that should be further elucidated in the future but it is likely a reason for the behavior we observed.

The next logical question is which specific viral components could be responsible for the defects we observed. Different HCV gene products have been shown to interfere with the normal function of DCs (52, 198). The structural proteins (core, E1/E2), a likely culprit for our results based on the lack of replication or entry, have been shown to interfere with monocytes and DCs by upregulating IL-10 or TNFα (dependent on the cell type and in a dose dependent manner), hindering IL-12 production and allogeneic T cell stimulation (48, 68). This was linked to a defect in the IFNα production by stimulated pDCs, which can also help induce IL-10 (63). The effects on pDCs may have been due to apoptosis induction while on mDCs and MoDCs may have been due to the interference with the cells differentiation into an active status (16, 35, 68, 189). Furthermore, the defective responses induced by these structural proteins can result in poor cellular and humoral responses (48). Therefore, the immunomodulatory role of HCV may lie at the level of DC differentiation.

An interesting caveat was that our DCs were able to produce cytokines in response to the virus even if these responses were skewed. This correlates with other studies that show that same phenomenon (48, 52, 61, 198). Furthermore, a similar pattern was observed in DCs where the viral proteins were expressed by AAV vectors, perhaps due to maturation by the AAV itself (59, 199). Still, this depends on the type of maturation stimuli as well as the state of infection of the patient from where the cells were obtained (chronic versus healthy) (16, 37, 52, 198). More so, matured DCs should be taken with caution as a higher CD83 and CD86 with lower HLA-DR expression (as we observed) has been seen in the mDCs of HCV infected patients which correlates with liver inflammation (49).

Two things that make myeloid DCs the focus of our studies are that 1) pDC can be 10-50 times less efficient than mDCs and MoDCs at expanding both $CD4^+$ and $CD8^+$ naïve cells; 2)

mDCs and MoDCs can secrete much higher levels of IL-12 which is important for shifting the balance towards a T_H1 response since T_H2 is associated with a chronic state of HCV infection; and 3) mDCs numbers are decreased in chronic patients that have not undergone anti-viral therapy also indicating the role of IFNα in maintaining DC populations (14-16, 47, 50, 124). Sometimes, it seems is not the decrease in the number of DCs in patients but a shift in the expression of HLA-DR complemented with lower $CD4^+$ T cell numbers (75). Monocytes are the main producers of IL-10 and TNFα, due to their numeric prevalence over DCs in blood, and these cytokines are known to inhibit the IFNα production by pDCs probably by inducing apoptosis (68, 70-72). In our system we found that HCV increased IL-10 production by monocytes and TNFα by mature DCs. Levels of IL-10 and TNFα are consistently higher in HCV infected patients and in DC after HCV infection and may be to blame for the apoptosis of pDC in infected patients (68). Recently, Shiina et al. and Ebihara et al. performed experiments similar to ours in which they found that direct interaction HCV does not affect matured mDCs or MoDCs (38, 73). While we show defects in MoDC this effects were more profound in immature DC or monocytes. Therefore, the maturation status of DC needs to be considered when studying the pathogenesis of HCV on these cells during infection. In conclusion, our results build upon recent reports show that HCV has a strategy to modulate the action of DCs by interfering with the type I IFN response and shifting to a T_H2 response and show the importance of studying the behavior of immature DC in HCV infection.

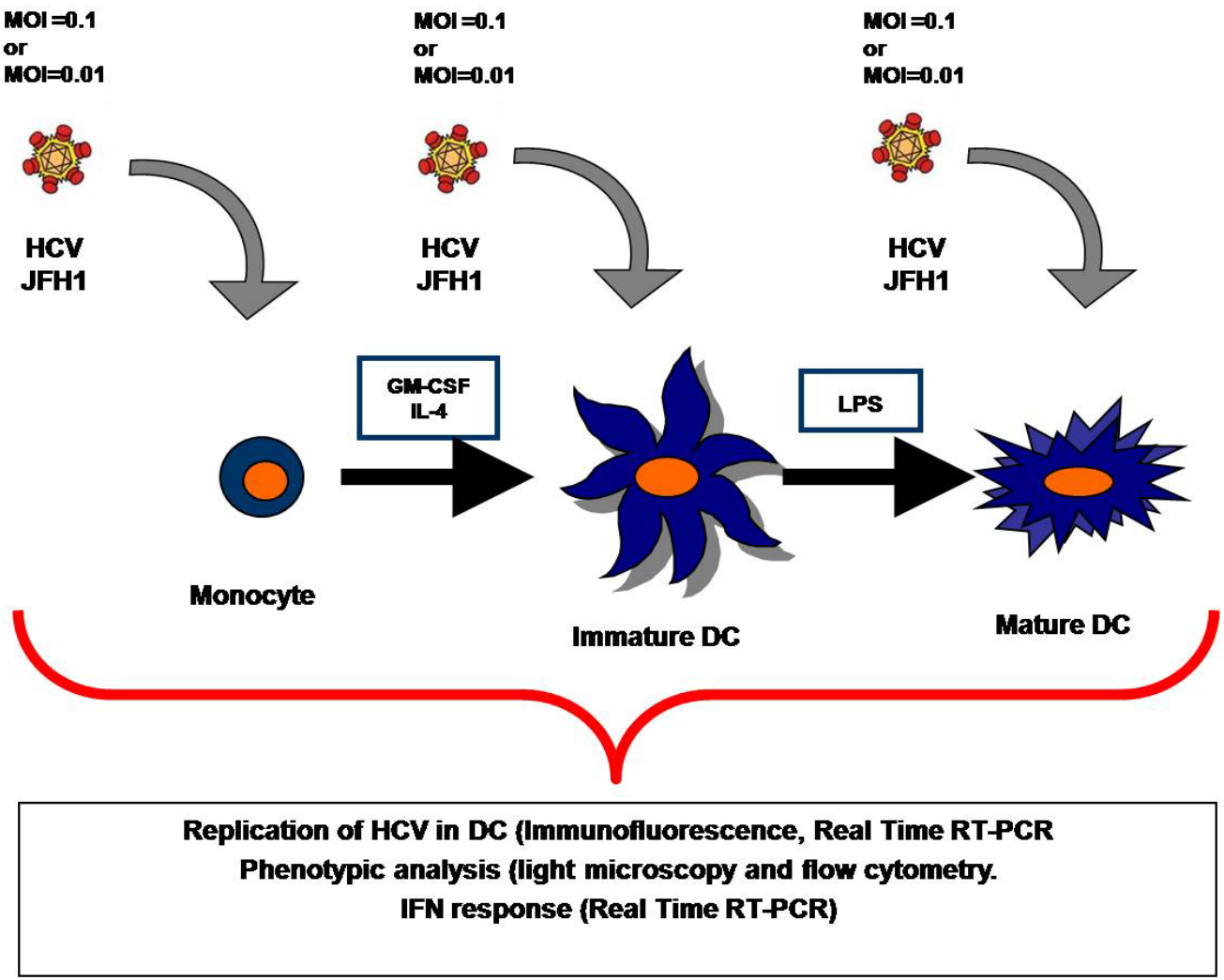


Figure 3-1. Experimental Approach to Study the Effects of HCV on Human Monocyte-Derived Dendritic Cells. Monocytes were isolated by adherence from healthy human PBMC. Immature DCs were obtained by culture of monocytes with GM-CSF and IL-4 for 6 days. Mature DCs were obtained by culturing Immature DC with LPS for 48 hours.Cells of each stage were incubated with HCV-JFH1, a genotype 2a strain of HCV that effectively replicates in culture at two different MOIs.

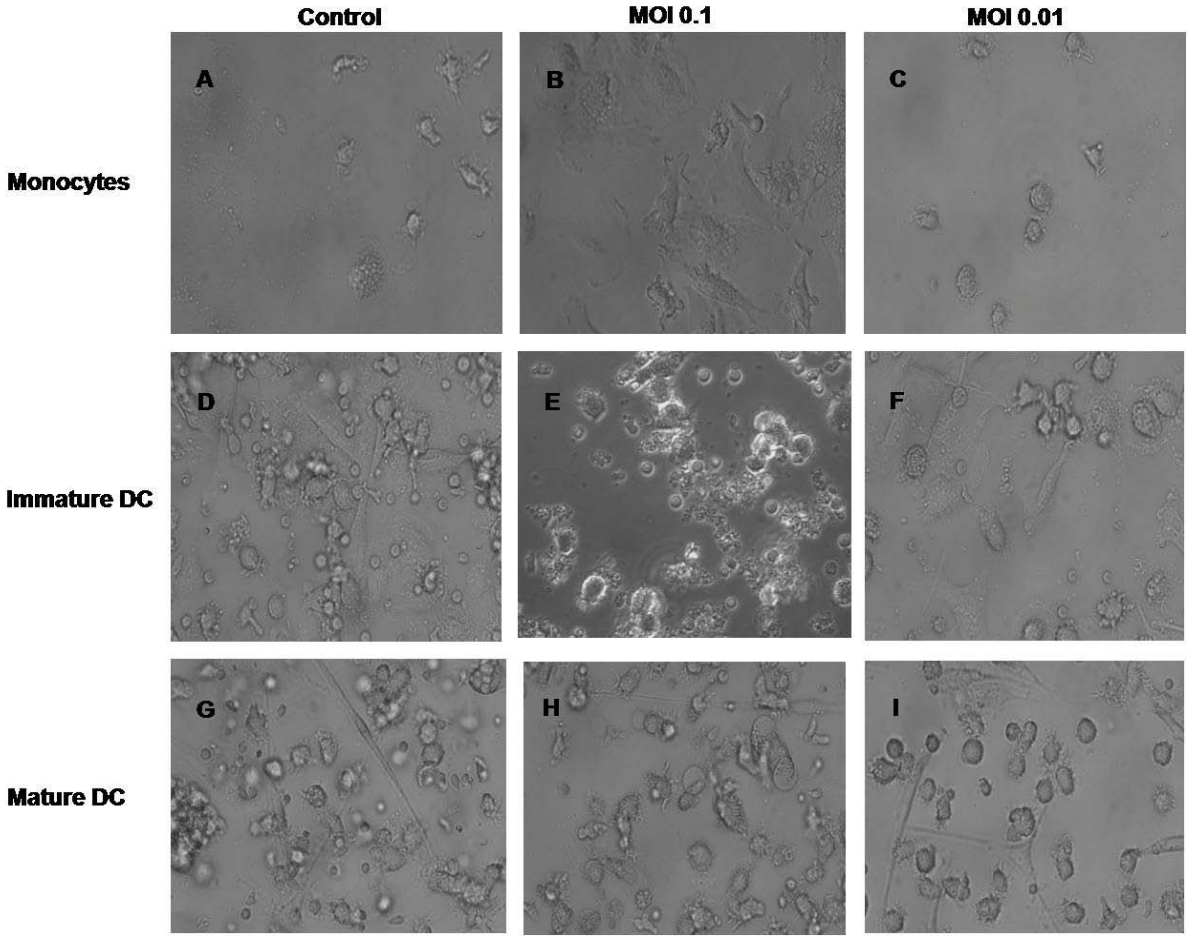


Figure 3-2.Morphology of Monocytes and Dendritic Cells Incubated with HCV-JFH1. Monocytes, immature DC and mature DC incubated with an MOI of 0.1 or an MOI=0.01 of HCV-JFH1for 7 days were observed at 100X magnification in a light microscope. Representative pictures of triplicate experiments (three separate donors) are shown. A) Control monocytes cultured without virus; B) monocytes incubated with MOI 0.1; C) monocytes incubated with MOI 0.01; D) immature DC without HCV; E) immature DC with MOI of 0 .1 of HCV; F) immature DC with an MOI of 0.01 of HCV; G) LPS matured DC without virus; H) LPS matured DC incubated with MOI of 0.1 of HCV; I) LPS matured DC incubated with MOI of 0.01.

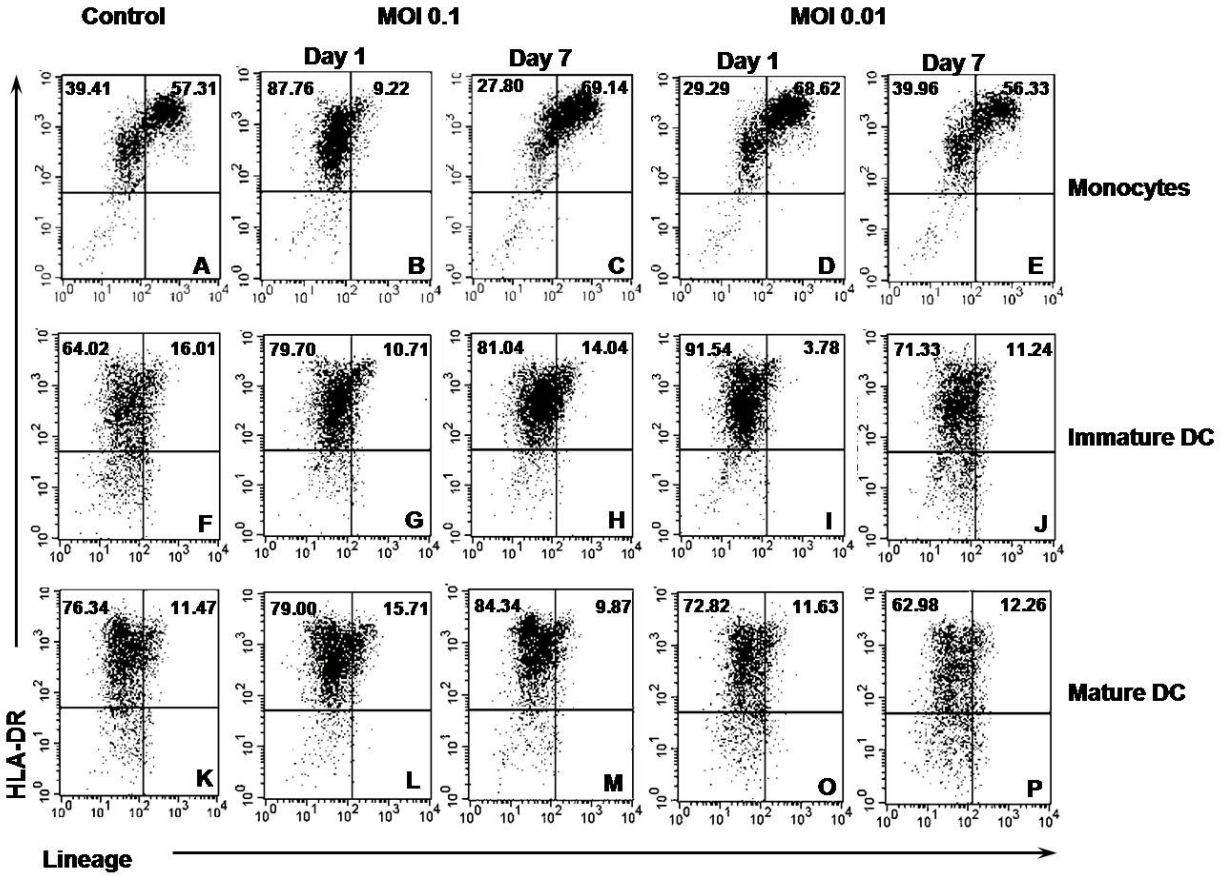


Figure 3-3. Phenotypical Characteristics of Monocytes and Dendritic Cells Incubated with HCV. Monocytes or DC from healthy human PBMC incubated with HCV for one or seven days were stained for HLA-DR and lineage 1 cocktail and analyzed by flow cytometry. The percentage of cells per quadrant from the total analyzed is shown. Figures are representative of three separate experiments (three separate donors). A through E) Monocytes. F through J) Immature DCs. K through P) Mature DCs.

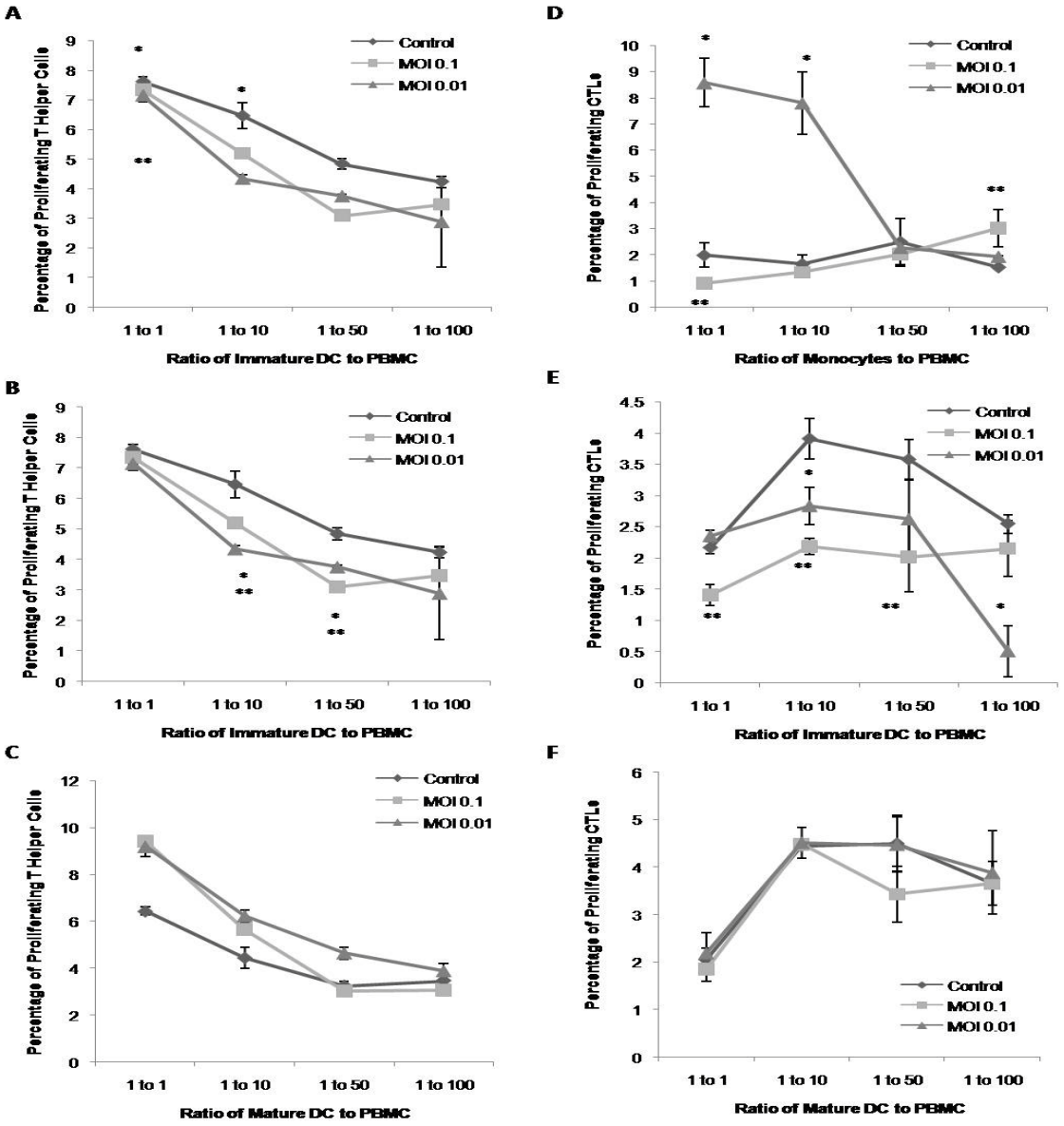


Figure 3-4. Immunostimulatory Capacity of Monocytes and Dendritic Cells Incubated with HCV. Monocytes or DC from healthy human PBMC incubated with HCV were co-cultured with autologous PBMC containing CFSE for seven days at 1:1, 1:10, 1:50 or 1:100 ratio of effector cells to proliferators. Cells were then also stained for CD3, CD4 and CD8, and analyzed by flow cytometry. Figures are representative of three separate experiments (three separate donors). A) Percentage of proliferating helper T cells ($CD3^+$ $CD4^+$) after stimulation with monocytes. B) Percentage of proliferating helper T cells after stimulation with immature DC. C) Percentage of proliferating helper T cells with mature DC. D) Percentage of proliferating CTL ($CD3^+$ $CD8^+$) after stimulation with monocytes. E) Percentage of proliferating CTL after stimulation with immature DCs. F) Percentage of proliferating CTL after stimulation with mature DCs.

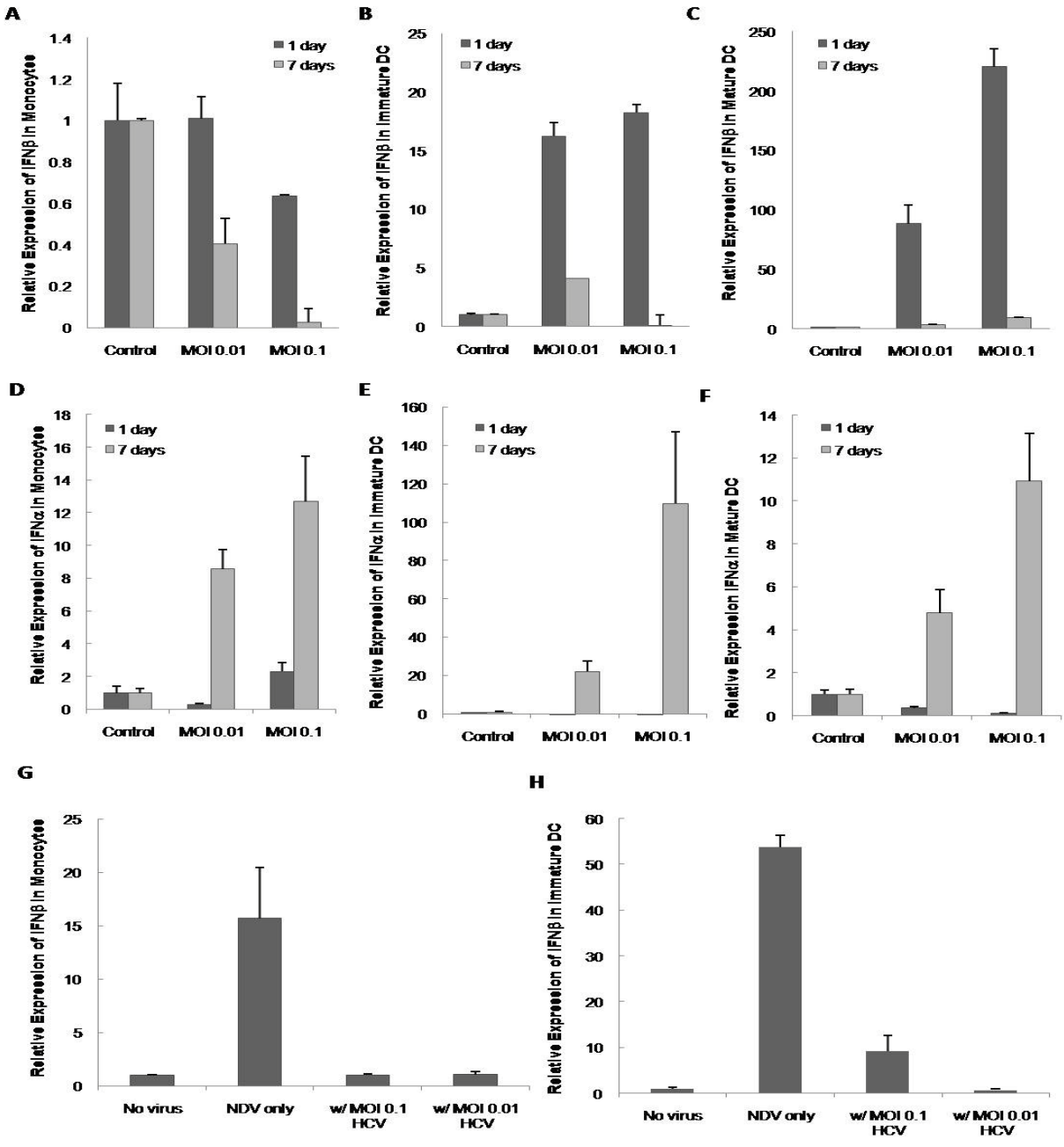


Figure 3-5. Type I IFN Production of Monocytes and Dendritic Cells Incubated with HCV-JFH. Monocytes (A and D), immature DC (B and E) and mature DC (C and F) were incubated with HCV and cells were collected at day 1 and day 7 post infection. Total RNA was isolated and IFN-β and IFNα2a mRNA expression was analyzed by real-time RT-PCR against unincubated controls and normalized against the housekeeping gene GAPDH. Monocytes (G) and immature DC (H) were either incubated by HCV alone or co-incubated with Newcastle Disease Virus (NDV). IFNβ was measured by Real Time RT-PCR. Bars represent the standard error (SEM) of three separate experiments (three separate donors).

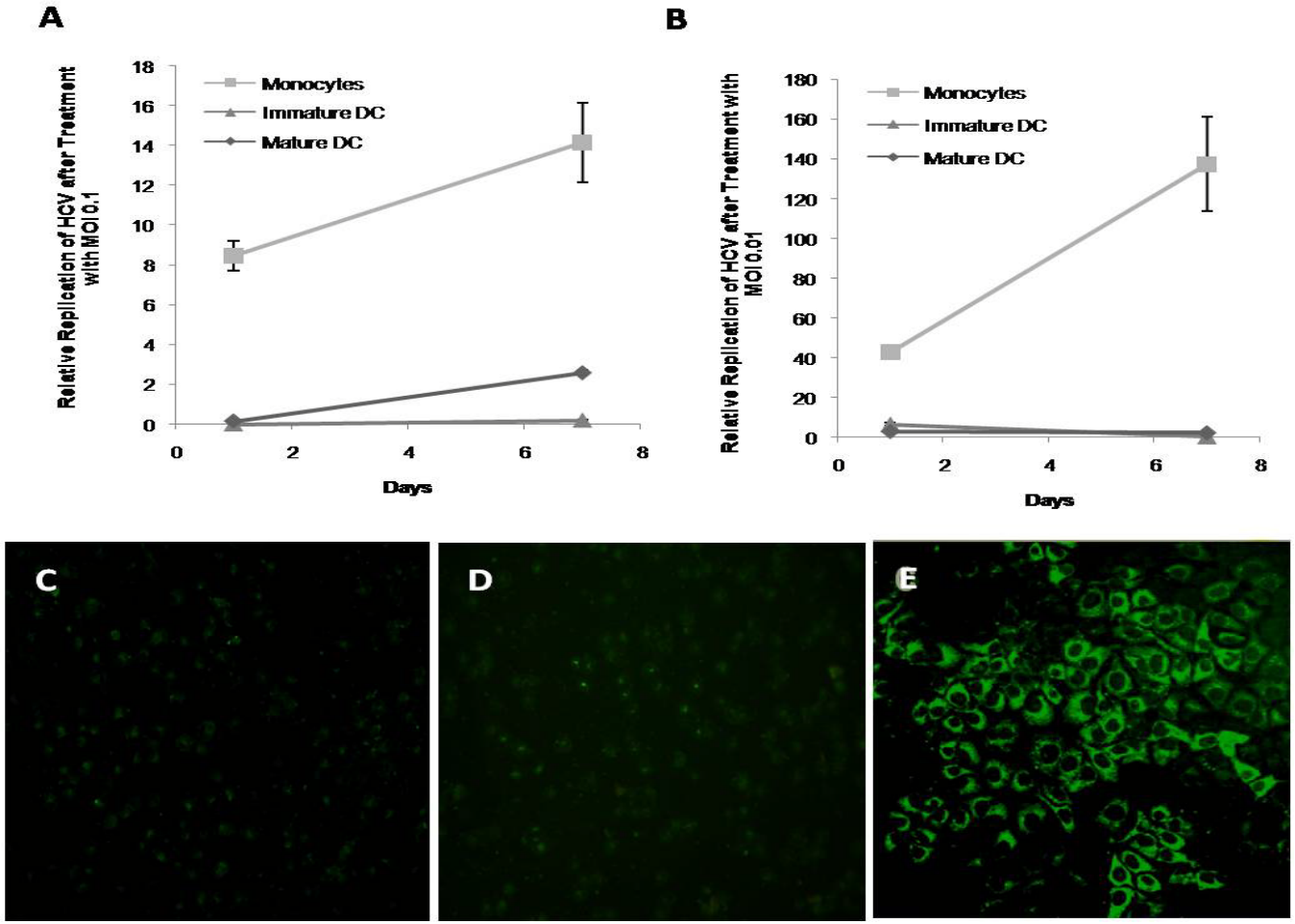


Figure 3-6. Lack of HCV Replication on Dendritic Cells Incubated with HCV-JFH1. Incubated cells were analyzed for HCV replication by immunofluorescence and by Real Time RT-PCR. A) Relative HCV expression in monocytes, immature DC and LPS matured DC after infection with MOI=0.1 of HCV-JFH1 particles. B) Relative NS5A expression in monocytes, immature DC and LPS matured DC after infection with MOI=0.01 HCV-JFH1 particles. C) Unincubated immature DCs (negative control) were stained with NS5A with FITC conjugated secondary antibody. D) Immature DC incubated with MOI=0.1 HCV. E) Huh 7.5 cells previously transfected with HCV-JFH1 plasmid (virus source, positive control).

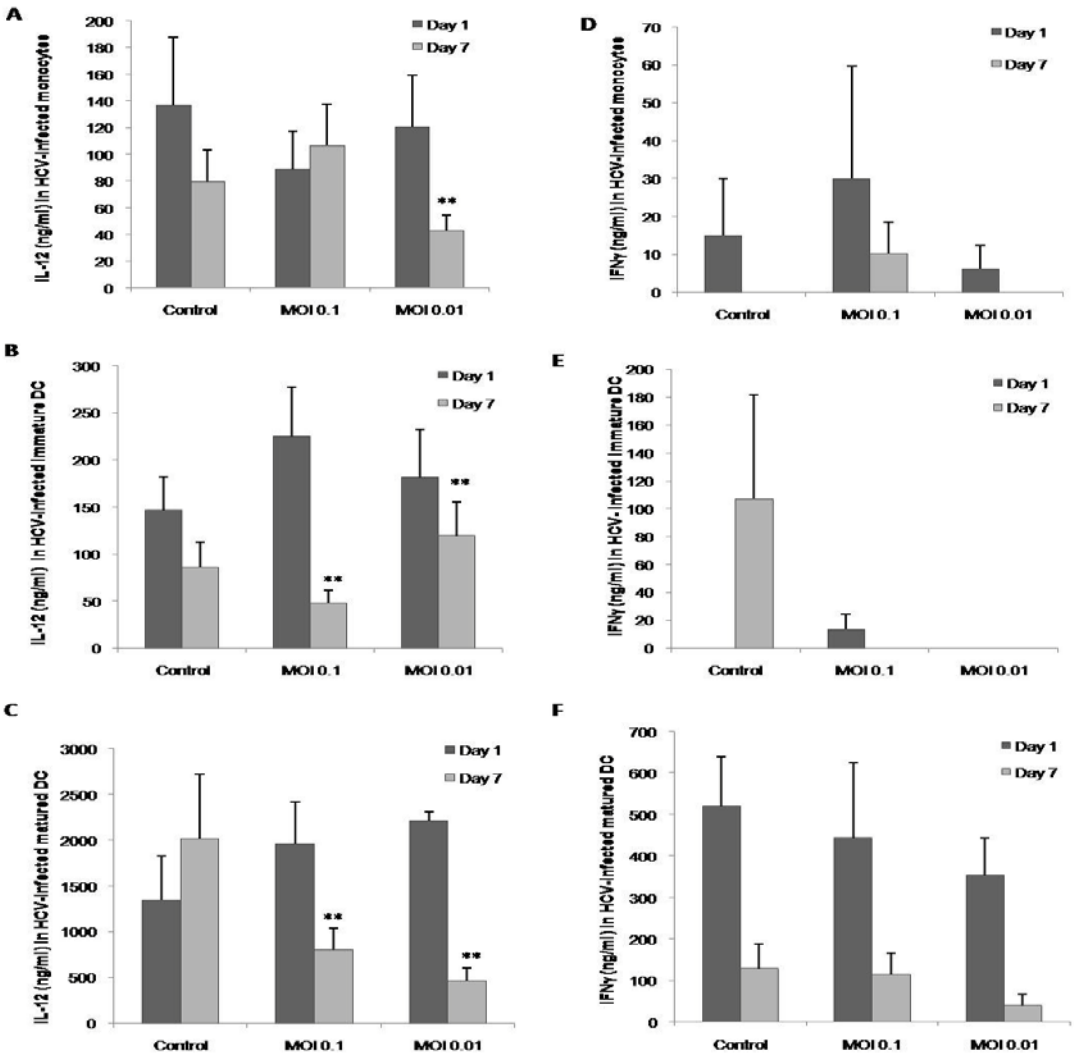


Figure 3-7. Concentration of TH1 cytokines IL-12 and IFNγ produced by monocytes and DC after HCV infection. Supernatant from monocyte, immature DC or mature DC cultures with or without HCV were collected at 1 and 7 days post infection and analyzed by capture ELISA. A) Concentration of IL-12 produced by monocytes. B) Concentration of IL-12 produced by immature DCs. C) Concentration of IL-12 produced by mature DCs. D) Concentration of IFNγ produced by monocytes. E) Concentration of IFNγproduced by immature DCs. F) Concentration of IFNγ produced by mature DCs. Bars represent the standard error (SEM) of three separate experiments (three separate donors). * represents the significance of the comparison between the day 7 control and the test in a paired student t-test where $p < 0.05$. ** represents the significance of the comparison between the day 7 control and the test in a paired student t-test where $p < 0.05$.

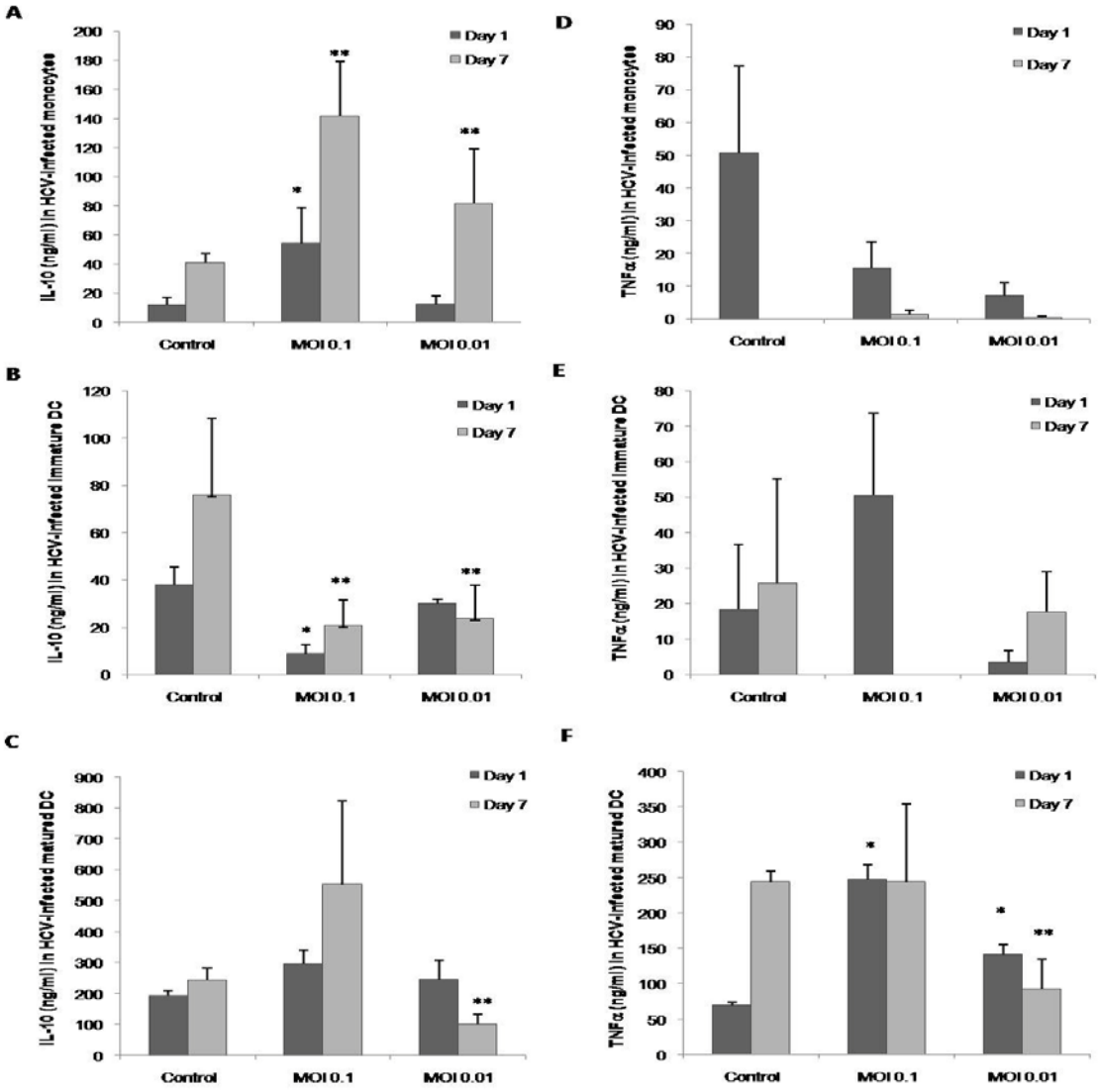


Figure 3-8.Concentration of IL-10 andTNFα produced by monocytes and DC after HCV infection. Supernatant from monocyte, immature DC or mature DC cultures with or without HCV were collected at 1 and 7 days post infection and analyzed by capture ELISA. A) Concentration of IL-10 produced by monocytes. B) Concentration of IL-10 produced by immature DCs. C) Concentration of IL-10 produced by mature DCs. D) Concentration of TNFα produced by monocytes. E) Concentration of TNFα produced by immature DCs. F) Concentration of TNFα produced by mature DCs. Bars represent the standard error (SEM) of three separate experiments (three separate donors). * represents the significance of the comparison between the day 7 control and the test in a paired student t-test where $p < 0.05$. ** represents the significance of the comparison between the day 7 control and the test in a paired student t-test where $p < 0.05$.

CHAPTER 4
DISRUPTION OF THE COOPERATION BETWEEN TLR3 AND RIG-I BY HCV'S STRUCTURAL PROTEINS

4.1 Materials and Methods

4.1.1 Cells Culture, Reagents and Plasmids

LH86 cells have been developed by our group from a resected, well-differentiated, hepatocellular carcinoma and Huh-7.5 cells were kindly provided by Dr. Charles M. Rice (Rockefeller University, New York, NY)(200). All cell lines were propagated in DMEM supplemented with 10% FBS, 200 μM L-glutamine, non-essential amino acids, penicillin, and streptomycin (complete DMEM or cDMEM). The expression vector pTOPO was from Invitrogen (Carlsbad, CA) and the negative control siRNA was produced by T 7 polymerase *in vitro* with the Ambion Silencer siRNA construction Kit or purchased (both control and kit from Ambion, Austin, TX). The plasmids for TLR3 and TLR7 (pUNO-hTLR3-HA and pUNO-hTLR7-HA) were purchased from Invivogen (San Diego, CA) and the siRNAs targeting those genes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Core, Envelope E1/E2 and NS3/4A were cloned using TOPO TA Cloning kit from Invitrogen (Table 1 shows PCR primers used for amplification).

Table 4-1.Primer sets used for cloning

Core	5'-TTGGATCCATGAGCACAAATCCTAAACC-3' 5'-CGTCTAGATCAAGCAGAGACCGGAACGGTGAT-3'
E1/E2	5'-GCTCTAGAGCCCAGGTGAAGAATACCAG-3' 5'-CGGGATCCTCATGCTTCGGCCTGGCCCAACAAG-3'
NS3/4A	5'-TTGGATCCATGGCTCCCATCACTGCTTATGCC -3' 5'-CGTCTAGATCAGCATTCCTCCATCTCATCAAAAGCC-3'

Cells were transfected using Lipofectin following the manufacturer's recommendations (Invitrogen). Briefly, in a 6-well tissue culture plate (Fisherbrand), 1 X 10^5 Huh7.5 or LH86 cells were seeded in 2 mL of cDMEM and incubated at 37°C overnight to allow for

reattachment. The next day 2ug of DNA diluted in a total of 20 uL serum free media and 20ul of lipofectin diluted to a 100 uL were incubated for 45 minutes at room temperature under a sterile hood before mixing and incubating for another 15 minutes under the same conditions. After the last incubation cells were mixed with serum free media to a total 1 ml volume and layered on top of prewashed adherent cells. The transfected cells were incubated for another 48 hours before changing into cDMEM at which point experiments were started. Stable cell clones were selected using antibiotics for a minimum of 4 weeks. All experiments were observed daily by light microscopy and cells collected for total RNA isolation with Trizol reagent (Invitrogen, Carlsbad, CA) unless otherwise specified in the results section. Poly (I:C) was obtained from InvivoGen (San Diego, CA).

4.1.2 HCV Constructs and Viral Particle Generation

pJFH-1 plasmid and pJFH-1/GND plasmid (negative control) were gifts from Dr. Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience, Toyko, Japan) (44). The linearized DNA was purified and used as a template for in vitro transcription using MEGAscript kit (Ambion, Austin, TX). In vitro transcribed genomic JFH-1 or JFH-1/GND RNA was delivered into Huh-7.5 cells by electroporation. The transfected cells were transferred to complete DMEM medium and cultured for the indicated period. Cells were passage every 3-5 days and corresponding supernatants were collected and filtered with 0.45 um filter device before freezing at -80oC. Viral titers were expressed as focus-forming units per milliliter (ffu/mL) and determined by the average number of NS5A-positive foci detected at the highest dilution of a serial dilution culture using Huh-7.5 cell line as host cells.

4.1.3 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

RT-PCR of total RNA to obtain cDNA was performed using the Superscript II (50 U reverse transcriptase per reaction) first-strand synthesis for RT-PCR kit (Invitrogen) primed with

oligo $(dT)_{12–18}$ (Invitrogen) according to the manufacturer's instructions. After reverse transcription, cDNA was used for quantitative real-time RT-PCR using fluorophore-labeled LUX primers from Invitrogen or SYBR (Table 2) some of which we have published before (201-203).

Table 4-2. Primer sets used for Real time RT-PCR(D-Lux primers show fluorochrome in the sequence)

HCV 3' UTR	5'-CCTTCTTTAATGGTGGCTCCAT-3' 5'-GGCTCACGGACCTTTCACA-3'
IFNβ	5'-CAGCCGATTCATCGAGCACTCGC[FAM]G-3' 5'-TTCCAGGACTGTCTTCAGATGG-3'
I-8U	5'CACGGTCATAGCATTCGCCTACTCCG[FAM]G-3' 5'GTCACGTCGCCAACCATCTT-3'
G1P3	5'-GACTGCTCGGAGGAGGACTCGCAG[FAM[C-3' 5'CAGGATCGCAGACCAGCTCA-3'
TRAIL	5'-ATGGTACCTCATGGCTATGATGGAGGTC-3' 5'-AAGCGGCCGCTCATAGTGTATCATCCTGAAAACTGA-3'
DR4	5'-GGGTCCACAAGACCTTCAAGT-3' 5'-TGGTGTAACCCACACCCTCT-3'
DR5	5'-AGAGGGATTGTGTCCACCTG-3' 5'AATCACCGACCTTGACCATC-3'
TLR3	5'-ACAACTTAGCACGGCTCTGGA-3' 5'-ACCTCAACTGGGATCTCGTCA-3'
RIG-I	5'-GACTGGACGTGGCAAAACAA-3' 5'-TTGAATGCATCCAATATACACTTCTG-3'
GADPH	5'-CGACCGGAGTCAACGGATTTGGT[JOE]G-3' 5'-GGCAACAATATCCAGTTTAGCA-3'

Reactions were conducted in a 96-well spectrofluorometric thermal cycler (StepOne Plus Sequence detector system, Applied Biosystems). Fluorescence was monitored during every PCR cycle at the annealing step. Results were analyzed with StepOne Plus software version 2.1 from Applied Biosystems. The PCR conditions were as follows: 50°C, 2 min; 95°C, 2 min (Super UDG master mix, Invitrogen) or 10 min (SYBR Green Master Mix, Applied Biosystems) and 40 cycles of 95°C, 15s; 60°C-62°C (depending on the primer set), 30s and 95°C, 1 min.

All genes were analyzed through the $2^{-\Delta\Delta Ct}$ method following previously described calculations (204). HCV copies were determined using instead a standard curve of JFH-1 full-length RNA transcribed *in vitro*. Results for all experiments represent triplicate determinations and are represented as means ±SEM.

4.1.4 Immuno-Fluorescence

Cells were grown on glass coverslips later to be washed and fixed on ice with 5% acetic acid in 100% ethanol (Fisherbrand). Cells were washed with 1X PBS and incubated with either goat anti-human TLR3 (Santa Cruz Biotechnology), goat anti-human TLR7 (Santa Cruz Biotechnology), rabbit anti-human RIG-I (ProSci Inc, Poway, CA) or mouse anti-HCV NS5A monoclonal antibody (established at our institution) for 1 hour. The respective secondary antibody (donkey anti-goat IgG FITC conjugated for TLR3 and TLR7, goat anti-rabbit IgG FITC conjugated, goat anti-mouse IgG FITC) were purchased from Southernbiotech (Birmingham, AL). Nuclei were counterstained with DAPI (Vector Laboratories Inc, Burlingame, CA), followed by examination under a fluorescence microscope (Olympus).

4.1.5 Western Blot Analysis

Cells were washed with PBS and lysed in RIPA buffer (150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris-Cl [pH 8.0] supplemented with 2 ug/mL of aprotinin, 2 ug/mL of leupeptin, 40ug/mL of phenylmethylsulfonyl fluoride, and 2 mmol/L DTT). Twenty μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules CA). The membranes were then blocked overnight at 4°C in blocking buffer (PBS containing 0.1% Tween 20 (PBS-T) and 5% fat-free milk power) followed by probing with each specific primary antibody (see immunfluorescence section for primary antibodies and

mouse anti-human β-actin from Santa Cruz Biotechnology) for one hour at room temperature. After washing thrice with PBS-T for 10 minutes each, the membrane was incubated with the appropriate HRP-conjugated secondary antibody (all from Santa Cruz Biotechnology), diluted in PBS-T, for 1 hour at room temperature and washed 3 more times. Proteins were visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc, Rockford, IL).

4.1.6 Flow Cytometry

Pelleted cells were incubated for 30 min at room temperature with antibodies (as in immunofluoresence section), washed with staining buffer (PBS + 2% BSA + 0.1% Na azide), and then fixed with 2% paraformaldehyde and stored at 4 °C. For intracellular staining the BDcytofix/cytoperm kit (BD biosciences) was used with the antibodies following the manufacturer's protocol. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Quantitation was done by the CellQuest software (BD Biosciences; version 3.2.1). Isotype-specific immunoglobulin controls were run for each fluorochrome. Thirty thousand cells were analyzed for each sample.

The Annexin V-FITC apoptosis was assayed as described previously (BD Pharmingen, San Diego, CA) (205). A minimum of 30,000 events per sample were acquired on a FACSCalibur Flow Cytometer (BD Pharmingen, San Diego, CA) and subsequently analyzed with CellQuest software (BD Biosciences; version 3.2.1, San Diego, CA).

4.2 Results

4.2.1 HCV Replication is Responsible for the Induction of IFN in LH86 Cells

We recently described the induction of IFNβ by the recently developed liver cell line LH86 in response to HCV (92). As described before, this cell line can serve as a model for the study of the initial interaction of HCV with hepatocytes and therefore we decided to use it to study the mechanisms behind this recognition. Our first question was to understand what incites

recognition by the host: genome or proteins. In order to do this we need to understand if replication of the virus is necessary to induce IFN. Therefore, we infected LH86 cells with HCV strain JFH-1 (genotype 2a) at a MOI of 1 and we also infected cells with the same amount of virus but heated for 15 minutes at 72^{o}C or exposed directly to UV light for the same amount of time. In this system, HCV induced a robust expression of IFNβ as long as it was replicating, since inactivating the virus by either treatment did not upregulate IFNβ (Figure 4.1A and 4.1B). Interestingly, when replication is lacking a strong downregulation of the baseline IFN level in these cells was observed which might highlight an immunomodulatory role of the virion itself.

In order to further understand the kinetics of this interaction we continued our experiments by culturing the cells with different dilutions of the same virus (lower MOIs). The IFN induction was titer dependent and lower dilutions of the virus not only did not induce IFN but also downregulated the basal levels as observed before perhaps indicating that a low level replication could be used by the virus to escape this response (Figure 4.1C and 4.1D). It might indicate and interplay between the host immunity and the virus evasion and further highlights the results from Figure 1A indicating that virion proteins may have an immunomodulatory effect. To corroborate that viral replication, and not RNA itself, is responsible for the induction of IFN, we electroporated LH86 cells with *in vitro* transcribed JFH-1 RNA of JFH-1/GND RNA. This virus has a mutation in its RdRp which prevents its replication but it is otherwise the same as the first (44). We found that JFH-1, and not JFH-1/GND, induced IFNβ in LH86 cells indicating the dependence of replication for its induction (Figure 4.1E and 4.1F). Furthermore, JFH-1/GND showed almost little downregulation of the basal IFN levels as compared to the other experimetns further indicating that the bulk of the immunomodulation is probably done by virion

proteins. This data shows interplay between the virus and liver cells in the early stages of an infection demonstrating that the perhaps the viral RNA can have strong IFN inducing capacity.

4.2.2 IFN Pathway in LH86 is Functional and PRR Expression is Comparable to Primary Hepatocytes

Recognition of the virus RNA can be done through several different PRRs. Most notable among those are the endosomal receptors TLR3 and TLR7 as well as the cytosolic receptor RIG-I all of which have been shown to be affected by the virus. Because the induction of IFN does not preclude that the elements further down this pathway are affected by the virus, we wanted to corroborate that the IFN produced by the virus was leading to the induction of ISGs. We then measured the expression of two ISGs: I-U8 and G1P3 in these cells with or without infection and got what we expected, a fully functional IFN response with the production of both these ISGs (Figure 4.2A and 4.2B). Many hepatic cell lines like Huh7 and derived cells have defects on these pathways at the level of the receptors by missing either one of them so we decided to characterize the expression of these receptors in LH86. This cell line expressed RIG-I and TLR3 strongly and as well as a mild expression of TLR7 as observed by immunofluorescence and further confirmed by flow cytometry (Figures 4.2C and 4.2D). These PRRs expression patterns compared to those in primary hepatocytes further highlighting the usefulness of these cells as a model for the study of HCVs IFN induction (Figure 2E and further confirmed by western blot, data not shown).

4.2.3 Both TLRs and RIG-I Share a Role in the Induction of IFN by HCV

These PRRs have been shown to have importance in HCV but their role in directly recognizing the virus or modulating its replication has not been fully categorized. We decided to do that by using LH86 and comparing it with Huh7.5 cells which lack all of these receptors and it is said to be likely the reason why they are so permissive for viral replication. Silencing TLR3

and 7 from LH86 cells abrogated the IFN response while adding those receptors to Huh7.5 induced it, albeit not to the same degree (Figure 4.3A and 4.3B). Furthermore, the presence of these receptors on Huh7.5 significantly downregulated the replication levels of the virus after a week (Figure 4.3C). Conversely, there was no clear change in the replication levels in LH86 after silencing of these receptors which may indicate that something else might be at play in the permissivity of the cell apart from the induction of IFN. It may also be due to the LH86 cells not being as permissive of viral replication as Huh7.5 is. While the removal of RIG-I showed a similar pattern to those of the TLRs by abrogating IFNβ, its addition only barely restored some IFN (Figures 4.3D and 4.3E). Interestingly, even though the levels of IFN were modest its addition to Huh7.5 cells the level of viral replication were also considerably reduced perhaps signaling that there is more to the role of RIG-I than just the induction of IFN. This data could also support the idea that TLR3 and RIG-I need to work together in order to induce enough IFN but that lack of this cytokine itself is not the only factor involved in the permissivity of Huh7.5 cells versus LH86.

These interesting results made us look further at the distinct effects that both TLRs and RIG-I can have. Apart from the induction of IFN, both pathways have also recently been described to be involved in the induction of apoptosis. From the experiments just described, we documented the changes in cell confluency by light microscopy before and after infection (Figure 4.3G, also corroborated by annexin V flow data not shown). Surprisingly, although TLR3 has been believed to be prominent for the induction of apoptosis and reducing it prevented cell death, it was the presence of RIG-I in both LH86 and Huh7.5 that correlated the best with apoptosis since silencing RIG-I prevented cell death while adding it to Huh7.5 induced it (TLRs

did not). These results may indicate that the cytopathic effects that we described for LH86 in response to HCV infection may be linked to the expression of RIG-I in these cells.

4.2.4 Cell Death through RIG-I in Response to HCV is Linked to the TRAIL Pathway

LH86 cytopathic effects are related to the induction of TRAIL and its receptors DR4 and DR5 therefore we studied the relationship between the PRRs and the expression of these three TRAIL pathway genes. As seen in figure 4.4, by day 4 of HCV infection silencing TLR3 and TLR7 in LH86 cells slightly decreased the expression of these three genes as compared to control cells. Conversely, while the TRAIL expression in RIG-I transfected cells was reduced only as much as that of TLR3/TLR7 silenced cells, the induction of both death receptors after HCV infection was strongly impaired by the silencing of RIG-I (Figure 4.4B and 4.4C).Interestingly this effect was not seen when RIG-I was added to the Huh7.5 cells since the addition of either receptor helped in the induction of these three genes by HCV. These results might indicate that the three receptors are involved in the induction of TRAIL pathway genes but RIG-I may have an important role in the maintenance or induction of the death receptors in the cells after HCV infection and this may hint as to why the transfection of RIG-I made the cells susceptible to the cytopathic effects of the virus.

4.2.5 TLR3 Can Control the Replication of the Virus with IFN but its Function in HCV Might Be Hindered by a RIG-I Crosstalk

Based on these results we defined that RIG-I is involved in cell death therefore TLR3's main goal in HCV infection is the induction of type I IFN responses important to create an antiviral milieu. Therefore we continue with a long term culture of stably transfected Huh7.5 cells with TLR3 or TLR7 and observe the replication of the virus and compare it to the IFN response. We infected the cells at the same MOI of previous experiments and continuously collected every 2-3 days for a maximum of 75 days and analyzed by Real Time PCR. As

expected both TLR3 and TLR7 had a profound effect on viral replication throughout the time of analysis, with TLR3 having the most profound effect (Figure 4.5A). By day 50 it seemed that HCV had been cleared from the cells but to our surprise it started rising again by day 54 albeit to very low copy numbers. This does not preclude that a longer culture could have restored the normal HCV replication level in these cells. TLR7 always had virus on it but seemed to have fluctuating levels of viral replication even if it was significantly lower than control cells. We had hypothesized that this effects are probably correlated with an IFN response which it did for both TLR3 and TLR7 (Figure 4.5B). TLR3 induced a very strong amount of IFN in the first week which did not come back again while TLR7 induced lower IFN which kept on coming throughout the culture and seems to correlate with lower copy numbers of HCV in those cells.

It seems then that TLR3 works in HCV by recognizing the virus an inducing a quick but strong IFNβ response which maintains the virus in check. Interestingly the virus did not clear from the cells indicating that perhaps an even stronger response might be needed for clearance. In any case, this highlights the importance of TLR3 in preventing viral replication and inducing IFN in response to HCV but opens to the question of what happens in a regular infection since both TLR3 and RIG-I would be present. The presence of both these receptors perhaps is needed to induce an even stronger IFN response or perhaps induce the death of the infected cells to clear the infection. In order to understand if these hold true for HCV we co-transfected the Huh7.5 TLR3 and TLR7 stable cell lines with RIG-I and PKR as a control. While TLR7 did synergize with RIG-I in the way we expected to induce a strong IFN response this did not happen with TLR3/RIG-I combination. In these cells, HCV infection at the same MOI as before did not induce IFN but actually downregulated it (Figure 4.5C). Co-transfecting with PKR did not

synergize or disrupt the IFN normally induced by either cell indicating that perhaps the effects are specific to TLR/RIG-I co-expression.

4.2.6 HCV's Envelope Proteins Downregulate the Expression of TLR3 and RIG-I

Based on these results we decided to examine more in detail the results we obtained at the beginning where we saw the downregulation of IFN from non-replicating virus (heated and UVd, Figure 4.1A). In order to do these we created stable cell lines with LH86 and three viral proteins: core, envelope (E1/E2) and NS3/4A as a control since we know that it is supposed to affect the IFN response by both RIG-I and TLR3 by cleavage of their adaptor proteins IPS-1 and TRIF respectively. After selection, cells were cultured with MOI=1 of the virus and the IFN response was measured daily for a week. LH86 NS3/4A showed a peak of IFN at day 4 just as before but much more reduced that in un-transduced cell which was expected and LH86 induced IFN most days but with an earlier peak (Figure 4.6A).

In contrast, LH86 cells that harbored the envelope proteins induced almost no IFN the first couple of days later to be reduced below basal levels after the midpoint of the culture. This would confirm initial suspicions that proteins in the virion are able to hamper the initial response of the cells and maybe involved in the induction of a favorable environment for viral growth.

The studies that have looked at NS3/4A and the initial interaction of the TLR and RIG-I pathways in HCV have demonstrated that the bulk of defects in these pathways are at the level of the adaptor proteins TRIF and IPS-1 or possibly above. Furthermore, clinical correlative studies have demonstrated that the levels of TLR3 and RIG-I are reduced in HCV patients therefore we wondered if the defects induced by the envelope proteins were related to these observations. HCV was able to reduce the level of TLR3 and RIG-I in apparently a titer dependent manner (Figure 4.6B). The virus proteins need to be intact for TLR3 downregulation which is demonstrated by the fact that heated virus was not able to downregulate it while RIG-I

downregulation needed intact virus for it to be downregulated (Figure 4.6C). Furthermore, the receptor downregulation is independent of each other and can be brought up by the induction of IFN since blots of Huh7.5 cells transfected with either receptor downregulated it after infection with an increase that correlated with the induction of IFN in those cells (Figure 4.6D).

In accordance with this, the levels of TLR3 were downregulated in LH86 cells carrying either one of the proteins we tested earlier but only NS3/4A continued to downregulate the levels of TLR3 when the cells were further infected with HCV (Figure 4.6E). Levels of RIG-I were also downregulated by the three proteins in these cells but interestingly it was only the envelope protein transfected LH86 that managed to keep those levels down indicating that the envelope proteins have a pivotal role in maintain RIG-I responses to a minimum at the beginning of the infection and maintain those levels low.

4.2.7 HCV's Envelope Proteins Affect the Induction of IFN to Cytosolic Stimulus

Our understanding is that the virus downregulates these receptor which in turn affect the overall response. In order to see if lower receptor levels correlate with lower IFN induction we decided to test LH86 E1E2 cells with poly I:C which is known to stimulate both TLR3 (when added directly to the media) and RIG-I (by transfection of poly I:C). As seen in Figure 7, both extracellular poly I:C and transfected poly I:C were able to induce a stale IFN response in these cells but while the presence of E1E2 affected the IFN response in cells stimulated extracellularly only for a day the presence of these viral proteins in LH86 cells completely abrogated the IFN response to poly I:C in these cells indicating that E1E2 can have a profound effect in the RIG-I pathway.

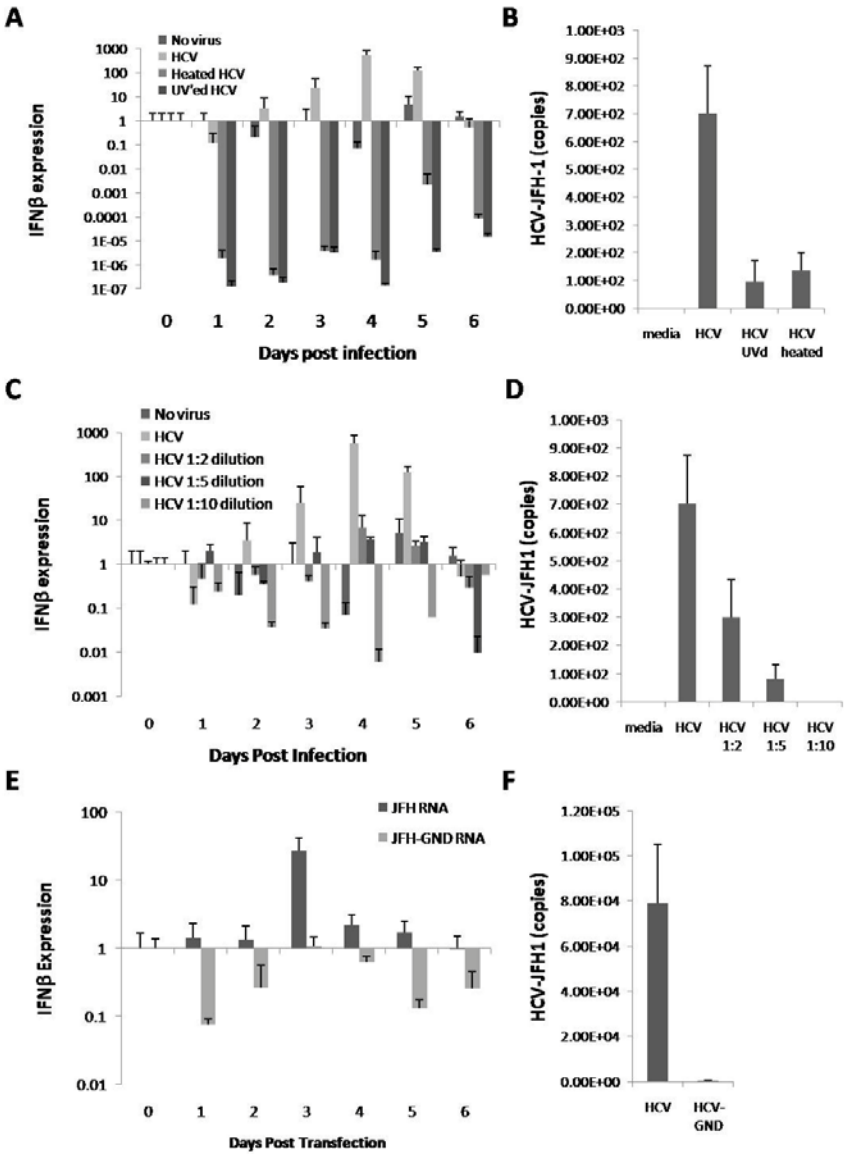


Figure 4-1. IFN response is dependent on viral replication. A) IFNβ expression of LH86 cells treated with HCV MOI of 1 and total RNA collected daily for gene expression. No virus indicates cells were cultured with the same volume in uninfected Huh7.5 supernatant, HCV is the supernatant from infected Huh7.5 cells as described in the methods section (MOI=1), heated HCV is the same superntant heated for 15 minutes at 72^{o}C and UVed HCV is virus that was exposed to UV light for 15 minutes under the culture hood. B) The replication levels measured at day 7 from the experiment described in A. C) IFNb expression of different dilutions of virus MOI=1. D) Replication levels at day 7 of the experiment described in C. E) IFNb expression of LH86 cells electroporated with *in vitro* transcribed HCV JFH1 or its non-replicating counterpart JFH-GND (day 0 is the day of the electroporation). F) Replication levels of experiment described in E at day 7.

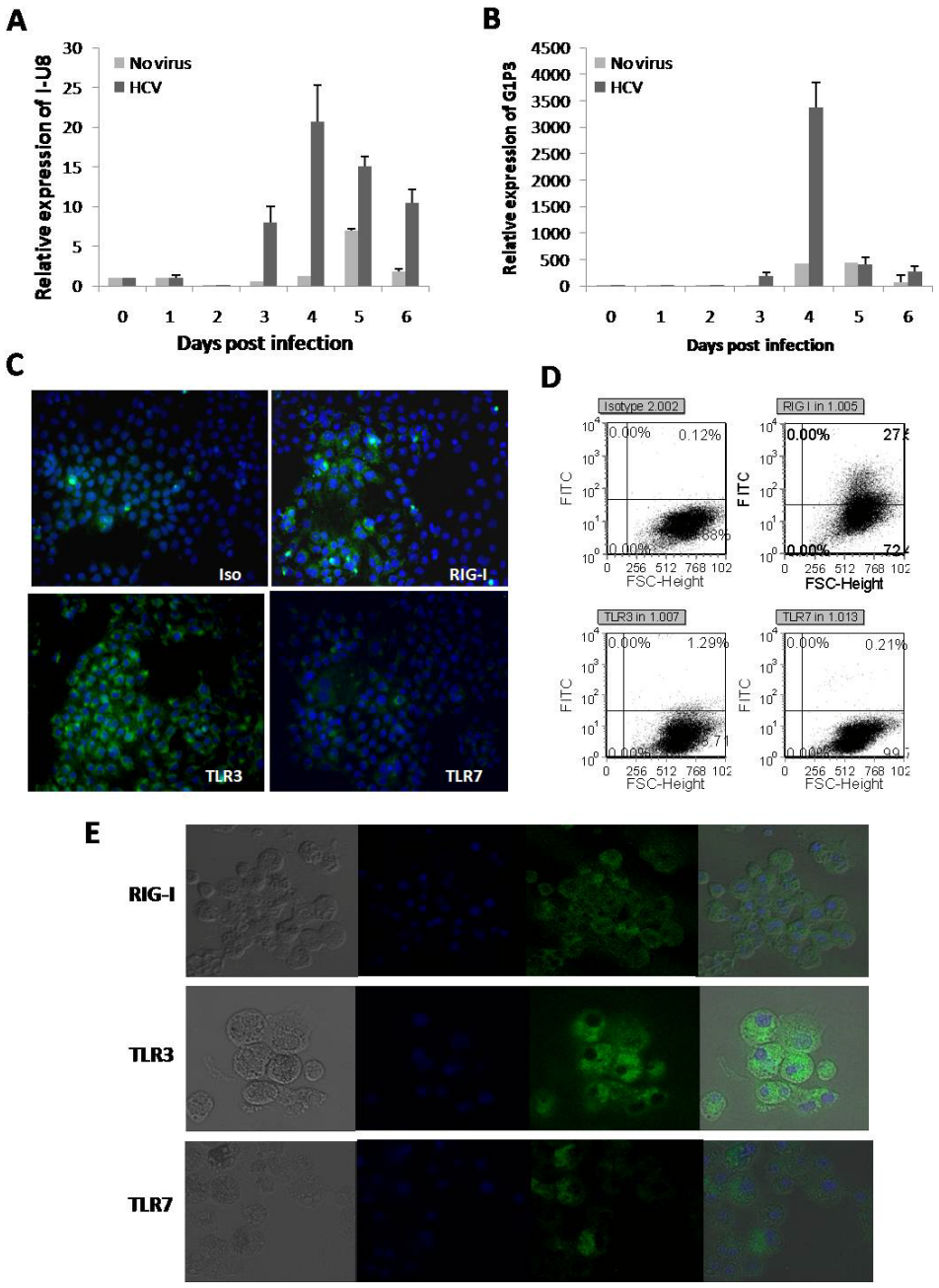


Figure 4-2. IFN pathway is responsive in LH86 cells. Experiments as described in figure 1 A) I-U8 expression in LH86 cells non-infected or infected with HCV-JFH-1 MOI=1. B) G1P3 expression in LH86 cells non-infected or infected with HCV-JFH-1 MOI=1. C) Expression of PRRs in LH86 cells: TLR3, TLR7 and RIG-I by immunofluorescence. Green is FITC and blue is DAPI. D) Extracellular and intracellular expression of PRRs in LH86 cells: TLR3, TLR7 and RIG-I. E) Expression of PRRs in primary human hepatocytes by immunofluorescence: TLR3, TLR7 and RIG-I. Green is FITC and blue is DAPI.

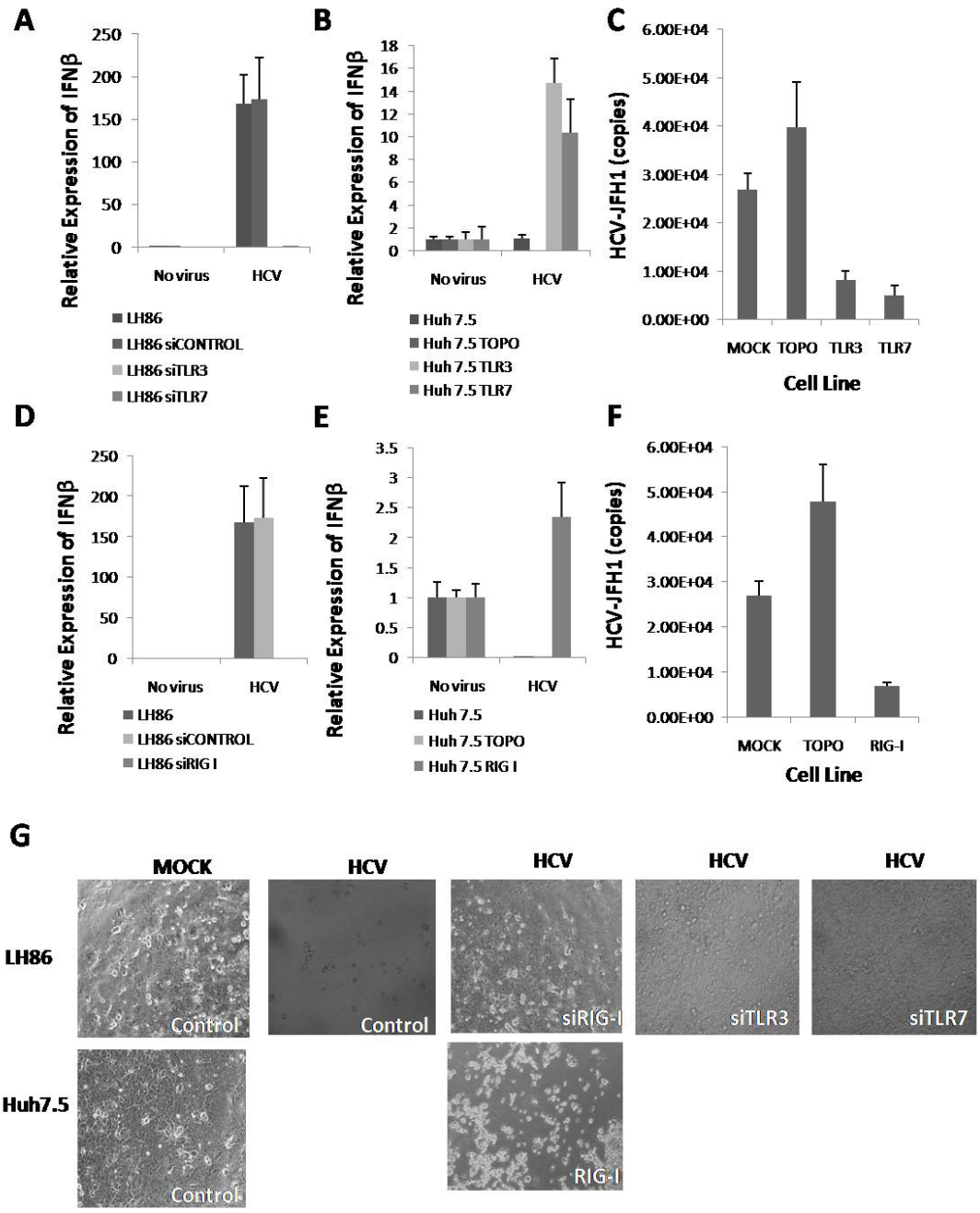


Figure 4-3. TLRs and RIG-I are both responsible for IFN but RIG-I is also responsible for cytotoxic effects of HCV. A) IFNβ expression (day 4 shown) in LH86 cells after silencing of TLR3 or TLR7. B) IFNβ expression in Huh7.5 cells expressing TLR3 or TLR7 (day 4 shown). C) Viral replication in Huh7.5 cells expressing TLR3 or TLR7 at day 7 after infection. D) IFNβ expression in LH86 cells with silenced RIG-I expression (Day4 shown). E) IFNβ expression in Huh7.5 cells expressing RIG-I (day4 shown). F) Viral replication in Huh7.5 cells transfected with RIG-I after 7 days of culture. G) Light microscope views of transfected cells after weeklong infection (cell denoted on the figure).

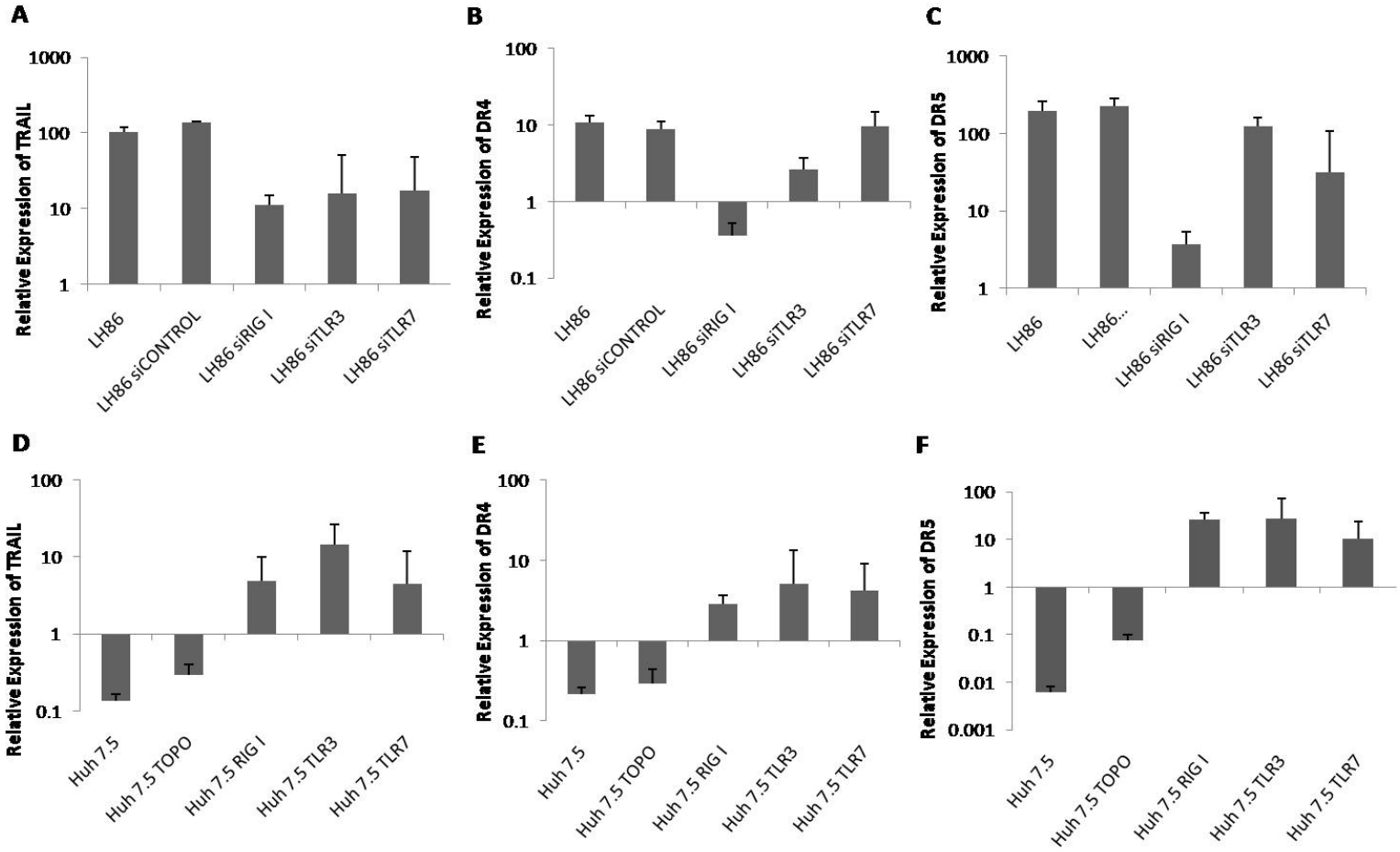
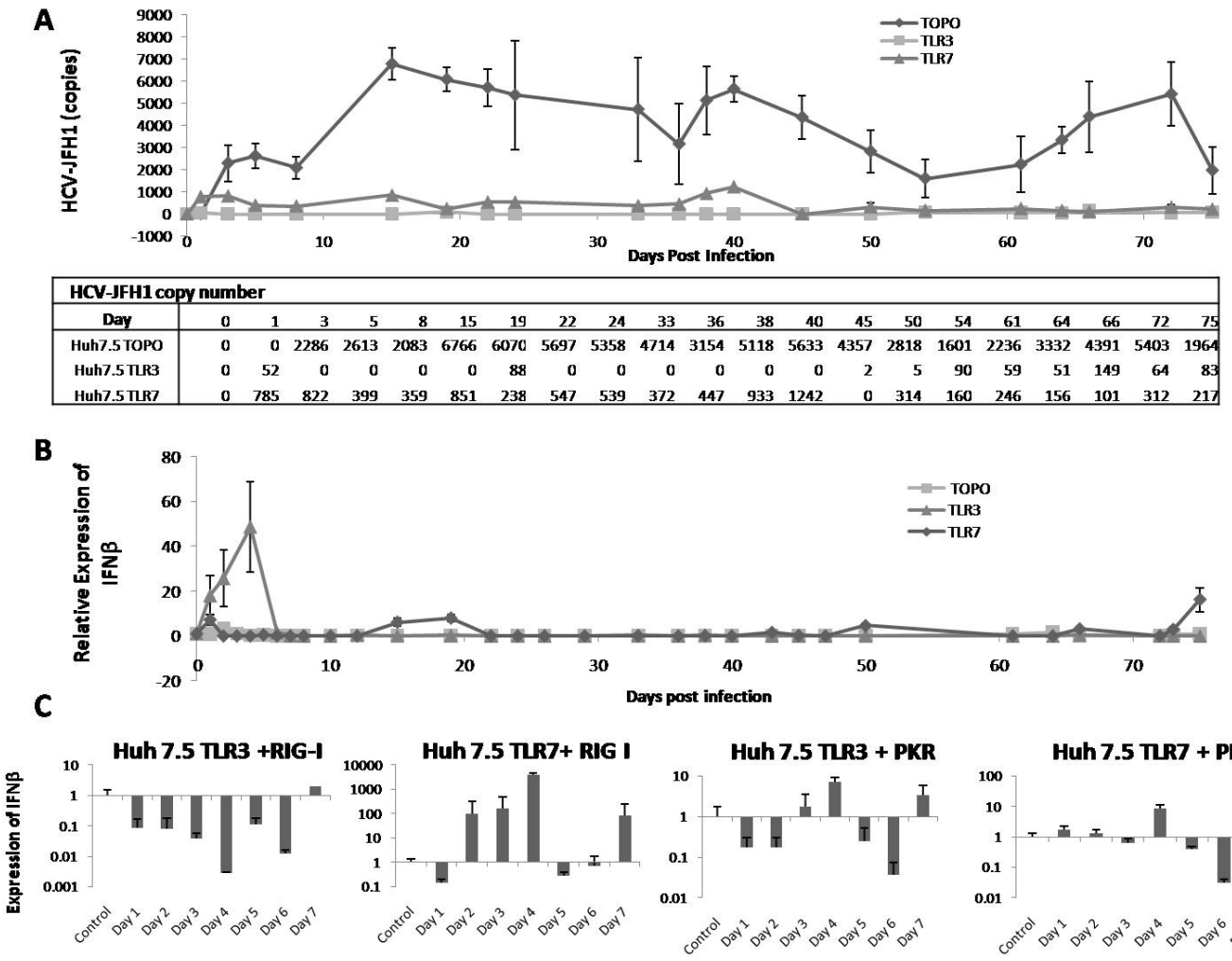


Figure 4-4. RIG-I is linked to the expression of TRAIL receptors DR4 and DR5. A) TRAIL expression in LH86 cells with silenced RIG-I TLR3 or TLR7 after infection (day 4 shown). B) DR4 expression in LH86 cells with silenced RIG-I TLR3 or TLR7 after infection (day 4 shown).C) DR5 expression in LH86 cells with silenced RIG-I TLR3 or TLR7 after infection (day 4 shown). D) TRAIL expression in Huh7.5 cells expressing RIG-I TLR3 or TLR7 after infection (day 4 shown). E) DR4 expression in Huh7.5 cells expressing RIG-I TLR3 or TLR7 after infection (day 4 shown). F) DR5 expression in Huh7.5 cells expressing RIG-I TLR3 or TLR7 after infection (day 4 shown).



HCV-JFH1 copy number

Day	0	1	3	5	8	15	19	22	24	33	36	38	40	45	50	54	61	64	66	72	75
Huh7.5 TOPO	0	0	2286	2613	2083	6766	6070	5697	5358	4714	3154	5118	5633	4357	2818	1601	2236	3332	4391	5403	1964
Huh7.5 TLR3	0	52	0	0	0	0	88	0	0	0	0	0	0	2	5	90	59	51	149	64	83
Huh7.5 TLR7	0	785	822	399	359	851	238	547	539	372	447	933	1242	0	314	160	246	156	101	312	217

Figure 4-5. TLR3 induces a strong initial IFN response but TLR3-RIG-I is affected by HCV preventing it. A) Viral replication in Huh7.5 cells stably transfected with TOPO (control) TLR3 or TLR7 infected with HCV MOI of 1 and collected every 2-3 days for RNA isolation (total 75 days). B) IFNb response of the experiment described in A. C) IFNb response in Huh7.5 stable cell lines cotransfected with either PKR and RIG-I after infection with HCV MOI=1.

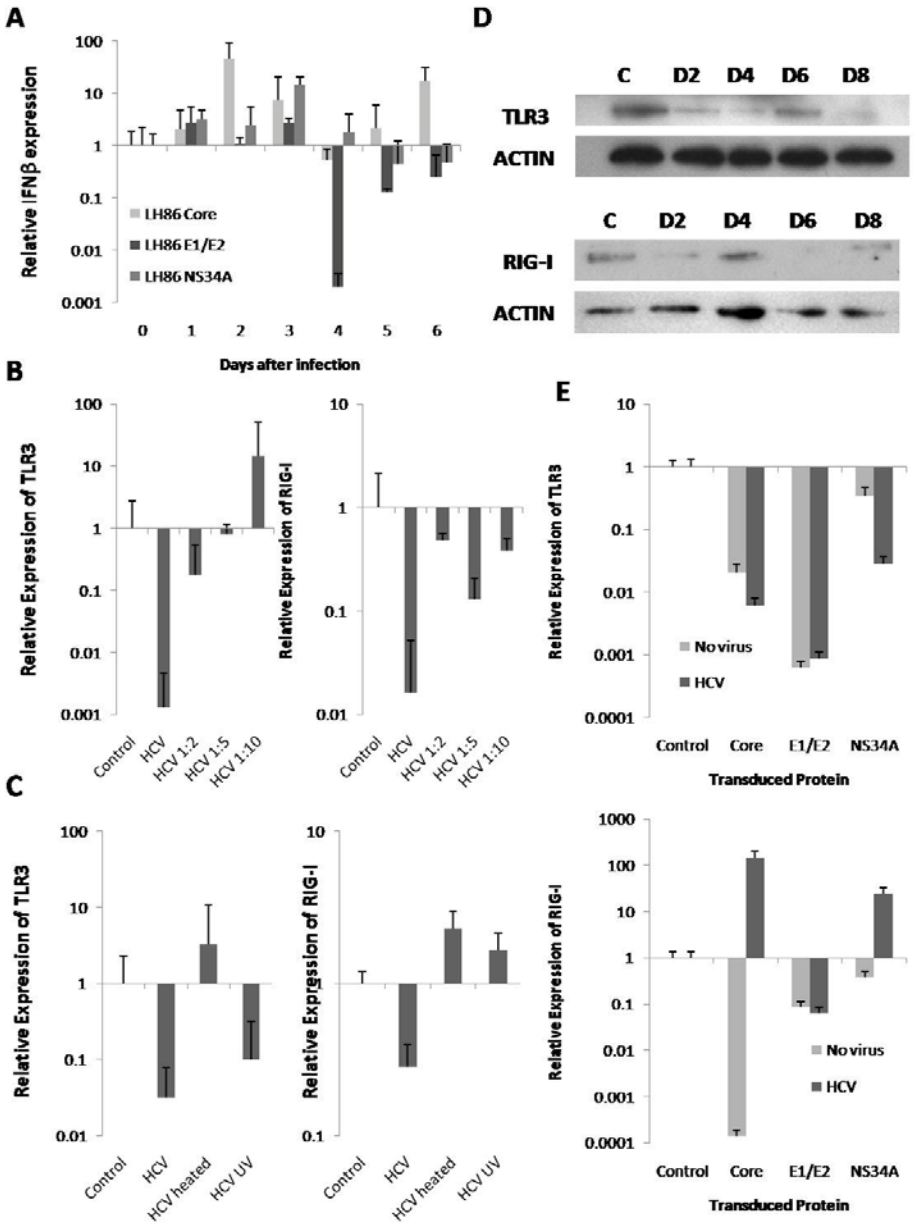


Figure 4-6. Virion proteins downregulate the expression of both TLR3 and RIG-I in hepatocytes. A) IFNb expression of stably transfected LH86 cells expressing Core, E1E2 or NS3/4A after HCV infection. B) TLR3 and RIG-I expression levels at day 7 after infection with different viral dilutions. C) TLR3 and RIG-I expression levels at day 7 after infection with normal, heated or UVed virus. D) Protein expression changes in Huh7.5 cells transfected with TLR3 or RIG-I after HCV infection. E) TLR3 or RIG-I expression in stably transfected LH86 cells carrying HCV proteins Core, E1/E2 or NS3/4A at day 7 after infection.

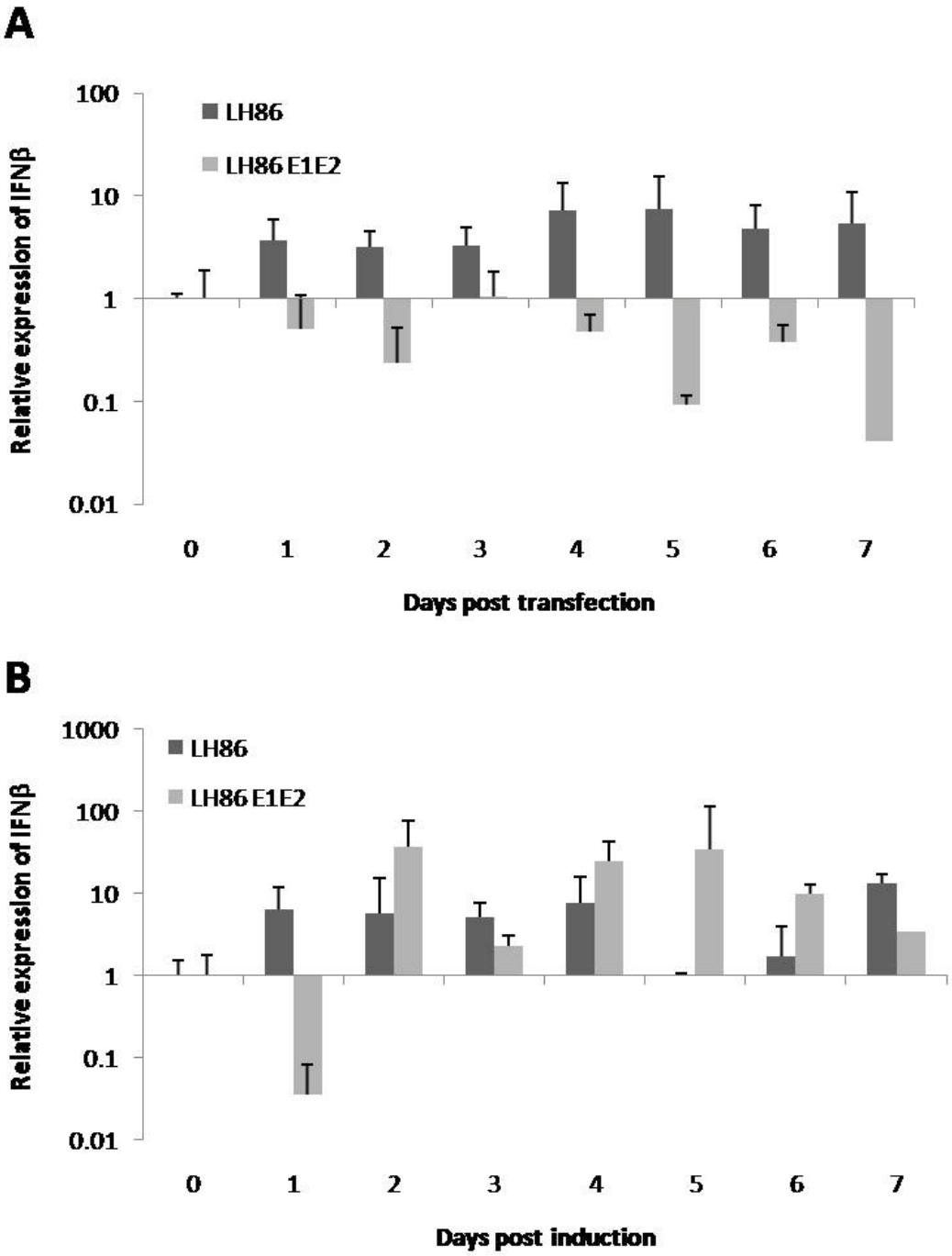


Figure 4-7. Envelope proteins affect the response to non-HCV responses through RIG-I receptor but not only temporarily through TLR3. A) IFNβ expression of LH86 cells or LH86 stably transfected with E1/E2 treated with 50ug/ul transfected Poly I:C. B) IFNβ expression of LH86 cells or LH86 stably transfected with E1/E2 treated with 50ug/ul extracellular Poly I:C.

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BIOGRAPHICAL SKETCH

Erika Adriana Eksioglu was born and raised in Caracas, Venezuela. She started her studies in biology at the Universidad Simon Bolivar also in Caracas. After two years she transferred to the University of Florida and entered the College of Agriculture to pursue a major in microbiology and cell sciences. She obtained her Bachelor of Science degree in December 1998.

After college she worked at the Department of Pharmacology at the University of Florida under Dr. Phillip Scarpace until September 1999. She then moved to Atlanta, Georgia, where she worked at Yerkes Primate Research Center for 3 years at the Tetramer Core Facility under Dr. John Altman and Dr. John Lippolis. In August 2003 she moved back to Gainesville to pursue her master's degree at the University of Florida in the College of Medicine. There she was under the mentorship of Dr. Vijay Reddy and worked on the role of growth factors in modulation of the immune response and on dendritic cell biology in the division of hematology/oncology. After wards she continued on with her graduate education to pursue a doctorate in immunology also at the University of Florida's College of Medicine. Under the tutelage of Dr. Chen Liu she studied, among other things, the innate immune response against HCV by liver cells. She plans to pursue an academic path in her future career endeavors.